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Direct benefits of ejaculate-derived compounds on female reproduction and immunity in the field cricket, Gryllus texensis

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Direct benefits of ejaculate-derived compounds on female reproduction and immunity in the field cricket, *Gryllus texensis*

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ABSTRACT

Sex consumes energy and drastically increases rates of disease, yet many female animals mate more than is necessary to ensure reproductive success. Surprisingly, this trend exists even in species in which the only resource that females receive from mating is the ejaculate itself. In this dissertation, I use the Texas field cricket, *Gryllus texensis*, to investigate how high mating rates enhance female fecundity and immune response, resulting in increased lifetime fitness. Using laboratory experiments, I examine the effect that mating multiple times and with multiple males has on female fecundity and survival. My results demonstrate that although the risks associated with mating are significant, the immediate fecundity benefits that females receive from high mating rates likely outweigh the costs.

Next, I investigate the sexual transfer and storage of prostaglandin E_2 (PGE_2), a component of the ejaculate that has long been hypothesized to be responsible for the increases in fitness that female crickets exhibit after multiple mating. My results confirm that PGE_2 fits the predictions required of it to be a likely candidate, as it is 1) transferred to the female spermatheca during copulation, 2) ephemerally available after mating, and 3) replenished and accumulated by females that mate multiple times. If ejaculate-derived PGE_2 truly acts as a fitness-enhancing compound providing direct benefits to females, it provides a proximate mechanism for why mated females experience increased fecundity and survival.

In the final portion of this dissertation, I experimentally test the effects of sexual maturity, copulation, accessory fluids, and testes-derived compounds on female fecundity and survival of a bacterial pathogen. My results demonstrate that testes-derived compounds are responsible for both inducing egg laying and up-regulating immune response in mated

female crickets. We posit that the acquisition of this limiting compound positively impacts female cricket physiology, thereby driving repeated mating events despite the energetic costs and disease risks of mating. These data reveal a potential biochemical mechanism to the end result of increased fitness achieved by females that mate frequently, providing a deeper understanding of how widespread multiple mating could evolve.

CHAPTER I

INTRODUCTION

Promiscuity is ubiquitous in nature and females often mate more than is necessary to ensure reproductive success; and yet, mate searching, courtship, and copulation can result in physical injuries, parasitism, predation, and disease (Arnqvist and Nilsson 2000), drastically reducing female survival and overall fitness. Unlike males whose fitness is often positively correlated with mating rate, females typically gain fitness by increasing their longevity to allow additional time to produce and care for more offspring (Bateman 1948; Trivers 1972) and should therefore minimize engagement in any activity that could result in premature death, including the act of mating. Why then, do females mate at such high rates?

Surprisingly, despite the costs of reproduction, mating multiple times generally increases female fecundity and offspring quality (Jennions and Petrie 2000; Wagner 2011), although the reasons leading to this increased fitness are varied across animal taxa. Multiple mating may increase overall fitness if females obtain indirect genetic benefits (Jennions and Petrie 2000), such as inbreeding avoidance (Hosken and Blanckenhorn 1999), increased genetic compatibility (Zeh and Zeh 1996; Tregenza and Wedell 1998), and increased offspring fitness via "good" genes (Slatyer et al. 2012). Alternatively, females may acquire resources from males that directly increase fecundity (i.e. the number of eggs produced), fertility (i.e. the number of viable offspring produced), or survival (Wagner 2011). These benefits include protection from predators (Rodríguez-Muñoz et al. 2011), nutritional gifts (Gwynne 1984; Fedorka and Mousseau 2002), or replenishment of viable sperm (Ridley 1988; Drnevich et al. 2001), but may also include ejaculate-derived compounds acquired for

use in essential physiological processes (Boggs and Gilbert 1979; Loher et al. 1981). Originally, ejaculates were thought to contain little more than sperm, but we now know that they are composed of myriad accessory substances that have profound effects on female physiology and behavior (Poiani 2006). If ejaculates contain fitness enhancing compounds, it may help explain why females that do not receive any of the other benefits listed above still mate at high rates.

Across taxa, the typical relationship between ejaculates and female fitness is that female fecundity significantly increases with ejaculate quantity, while lifespan is decreased (South and Lewis 2011). In contrast to this pattern however, female Orthoptera (i.e. crickets, grasshoppers, weta, and locusts) that receive more ejaculates experience both significantly higher fecundity and longevity (South and Lewis 2011). In the cricket *Gryllus lineaticeps*, a 32% increase in longevity was observed in multiply-mated females relative to once-mated females (Wagner et al. 2001). The functional mechanism for this longevity increase after mating is unknown, but there is evidence suggesting that ejaculatory products boost immunocompetence, thereby increasing survival. This has been demonstrated in crickets, where mated *G. texensis* survive bacterial infections better than virgins (Shoemaker et al. 2006). Although these studies suggest that direct benefits acquired through the ejaculate are responsible, the molecular and/or biochemical underpinnings accountable for these changes is unknown.

One candidate substance is prostaglandin, a biologically active metabolite of the C_{20} polyunsaturated fatty acid, arachidonic acid and other C_{20} polyunsaturated fatty acids. Prostaglandin is ubiquitous in animal taxa and mediates a large array of biological processes. In insects, its primary functions include egg production, oviposition, cellular immune

defense, ion/water transport, and thermoregulation (Craig 1975; Wolfe and Coceani 1979; Stanley-Samuelson 1994; Harris et al. 2002). It is likely in high demand because it is essential in regulating so many physiological processes within a single organism. Interestingly, most insects must obtain arachidonic acid from the diet since few are capable of synthesizing it *de novo* (Blomquist et al., 1991), however some male crickets retain this ability, thereby providing them with a non-dietary source of these essential compounds. If males transfer prostaglandin to females during mating, this may explain the increased fecundity (via increased egg production and oviposition) and survivorship (via a boost in immunity) females experience, as well as their tendency to mate multiple times if all males are providing an otherwise limited nutrient in the ejaculate.

Indeed, many studies have shown the presence of prostaglandin and prostaglandin precursors in the reproductive tissues and ejaculates of crickets (Destephano et al. 1974; Loher et al. 1981; Stanley-Samuelson and Loher 1983; Stanley-Samuelson et al. 1983). The testes provide sperm, prostaglandin, and the enzyme complex prostaglandin synthetase (Tobe and Loher 1983); alternatively, the testes and the accessory glands provide the substrate arachidonic acid (Stanley-Samuelson et al. 1987). These components are packaged into a spermatophore by the male and transferred to the female during mating. The female uses these components to synthesize additional prostaglandin after copulation is completed (Figure 1), and prostaglandin is then transferred to the genital chamber where it can be used to elicit various physiological processes (Stanley-Samuelson 1987).

Empirical research has indicated that prostaglandins play a large role in cricket reproduction. Studies on two species of Orthoptera revealed an increase in oviposition after an injection of prostaglandin (Loher et al. 1981; Stanley-Samuelson et al. 1986). The increase

in oviposition is dose-dependent, such that the number of eggs laid is positively correlated with the amount of prostaglandin injected (Destephano and Brady 1977). This effect of prostaglandin-injection on egg laying is similar to the effect of mating on oviposition rates (Loher and Edson 1973; Murtaugh and Denlinger 1985), which could be explained by prostaglandin that is transferred in the spermatophore. In fact, virgin females that have a spermathecae from mated females placed into their body cavities exhibit increased oviposition (Ai et al. 1986), suggesting that changes in oviposition after mating are due to the contents of the ejaculate and not the mechanical stimulation of mating.

In addition to its effects in reproduction, prostaglandin is a major modulator of cellular and humoral immunity in a broad array of insect taxa (Stanley et al. 2009). This has been demonstrated in numerous insect taxa, where the use of various prostaglandin synthesis inhibitors has a dose-dependent effect on immunity, with larger doses resulting in reduced immunocompetence; however, the immune response can be rescued with an injection of prostaglandin or arachidonic acid directly into the hemolymph (Mandato et al. 1997; Jurenka et al. 1997; Morishima et al. 1997; Bundey et al. 2003; Miller 2005). Only one such study has focused on crickets, but the study supported prostaglandin's role in immunity, revealing that individuals injected with prostaglandin-inhibitors had a significantly reduced nodulation response, an important cellular immune defense for clearing bacterial infections (Miller et al. 1999).

Both reproduction and survival determine overall female fitness, such that a decline in either could drastically reduce an individual's lifetime reproductive success. Although considerable research has demonstrated the role that prostaglandins play in mediating oviposition and immunity, the broader impacts prostaglandins have on female fitness and the

evolution of multiple mating has yet to be investigated. The acquisition of large amounts of prostaglandin via mating may provide insight not only into post-mating changes in female physiology, but also indicate a mechanism for how these important fitness traits are maintained and an answer to why females mate multiple times when sex is associated with high fitness. Crickets are emerging model organisms for these types of investigations because the current knowledge on their immunity, reproduction, and life history is vast, allowing for in-depth studies on mechanisms of increased fecundity and survival induced by mating that drive the evolution of high female mating rates.

In this dissertation, I use the Texas field cricket, *Gryllus texensis*, to examine the fitness benefits that females gain from mating at high rates in species that do not receive material resources, parental care, or protection from predators from their mates. In Chapter 2, I evaluate both the survival costs and fecundity benefits associated with multiple mating and ejaculate replenishment in female *G. texensis.* In Chapter 3, I explore the role that a specific component of the ejaculate, prostaglandin E_2, could have on increased fitness that females experience after mating at higher rates. In Chapter 4, I experimentally identify which aspect of mating results in the increased immune response of mated female crickets and narrow down the multitude of components comprising the ejaculate to a single candidate that is likely responsible for these post-mating changes in female physiology. Finally, the results from earlier chapters are summarized in Chapter 5 where I discuss the evolution of female multiple mating.

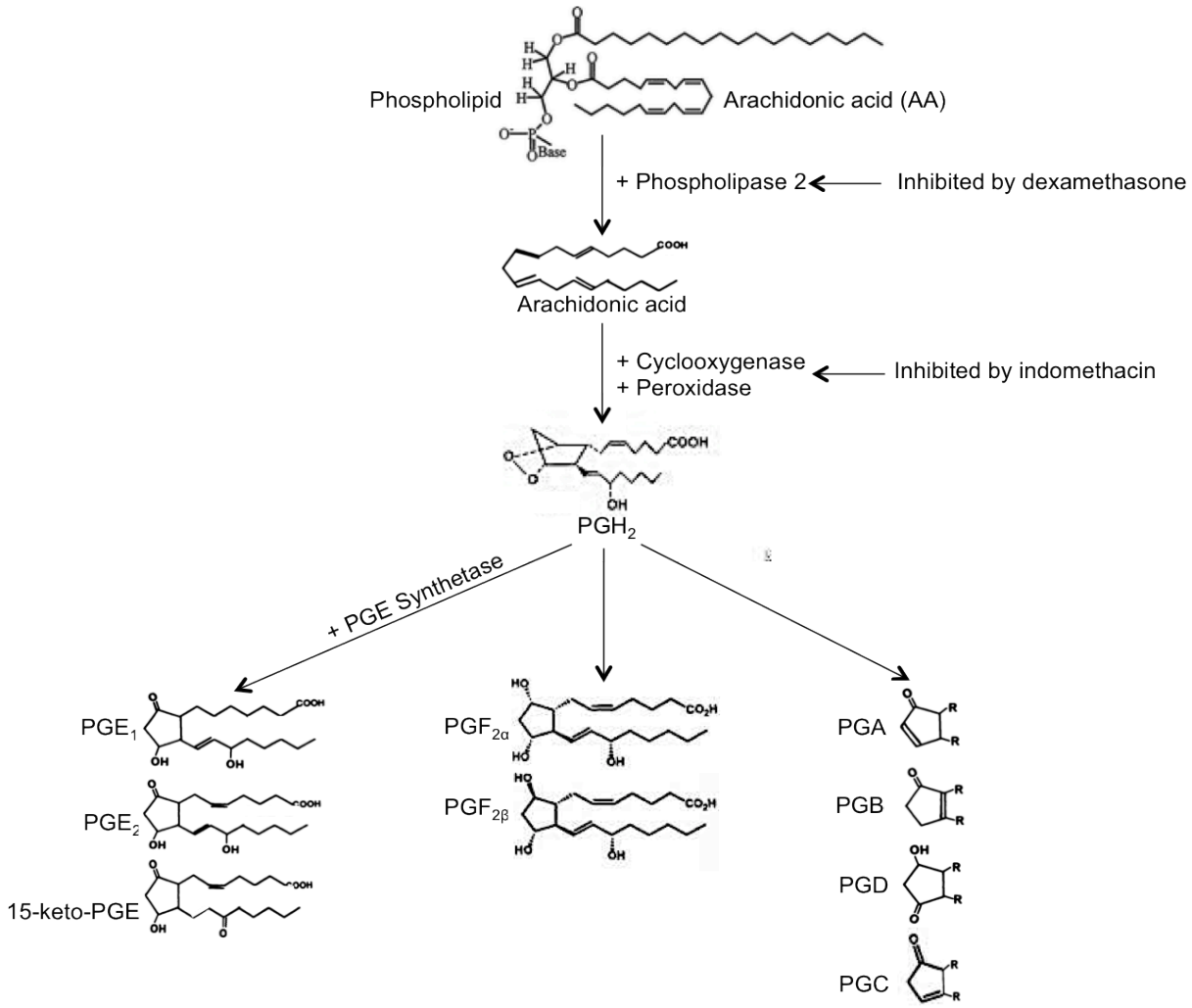

Figure 1: Prostaglandin biosynthesis begins by phospholipase 2 hydrolyzing polyunsaturated fatty acids into arachidonic acid. Cyclooxygenase and peroxidase then convert arachidonic acid into PGH_2, which can be transformed into one of the many forms of prostaglandin. For example, PGE synthetase is responsible for the formation of E-series prostaglandins.

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CHAPTER II

DIRECT COSTS AND BENEFITS OF MULTIPLE MATING: ARE HIGH FEMALE MATING RATES DUE TO EJACULATE REPLENISHMENT?

A paper submitted for publication to a peer-reviewed journal
Amy M. Worthington & Clint D. Kelly

Abstract

Females often mate more than is necessary to ensure reproductive success even when they incur significant costs from doing so. Direct benefits are hypothesized to be the driving force of high female mating rates, yet species in which females only receive an ejaculate from their mate still realize increased fitness from multiple mating. Here, we experimentally test the hypothesis that multiple mating via monandry or polyandry increases female fitness by replenishing ejaculates, thereby allowing females to produce more offspring for a longer period of time. We found that higher rates of female mating significantly increased lifetime fecundity independent of whether females mated with one or two males. Further, although interactions with males significantly increased rates of injury or death, females that replenished ejaculates experienced an increased rate and duration of oviposition, demonstrating that the immediate benefits of multiple mating may greatly outweigh the long-term costs that mating poses to female condition and survival. We suggest that ejaculate replenishment is a driving factor of high mating rates in females that do not receive external direct benefits from mating and that a comparative study across taxa will provide additional insight into the role that ejaculate size plays in the evolution of female mating rates.

Introduction

Sex consumes time and energy, can result in physical injuries, and drastically increases rates of parasitism, predation, and disease (reviewed in Arnqvist and Nilsson 2000). In addition to these costs, female fitness is generally limited by the number of eggs produced and not by access to males (Bateman 1948; Trivers 1971), yet females of many animal species mate more than is necessary to ensure reproductive success (Simmons 1988, Jennions and Petrie 2000). Although male coercion may provide an explanation of why females mate more than is optimal (Arnqvist and Rowe 2005), many qualitative (Zeh and Zeh 2001; Simmons 2005; Rhainds 2010) and quantitative (Arnqvist and Nilsson 2000; South and Lewis 2011; Slatyer et al. 2012a) reviews have compiled evidence suggesting that females benefit from mating more than once. Multiple mating may increase overall fitness if females obtain indirect genetic benefits by mating with several males (i.e. polyandry) within a given reproductive cycle (reviewed in: Jennions and Petrie 2000; Zeh and Zeh 2001). Such benefits include inbreeding avoidance (Hosken and Blanckenhorn 1999), increased genetic compatibility (Zeh and Zeh 1996; Tregenza and Wedell 1998), and increased offspring fitness via "good" genes (Slatyer et al. 2012a) or heterozygosity at fitness enhancing loci (Brown 1997). While many empirical studies support the indirect genetic benefits hypothesis, a recent meta-analysis (Slatyer et al. 2012a) found that the effect of polyandry on female fitness throughout animal taxa is relatively small and therefore unlikely to be the driving force behind the high rates of mating by females that are observed across taxa.

Alternatively, mating multiple times may increase female fitness if males provide direct benefits that boost female lifetime fecundity or survival (reviewed in (Wagner 2011). These benefits can take the form of protection from predators (Rodríguez-Muñoz et al. 2011),

defense against harassing males (Cordero 1999), assistance in defending resources (Slatyer et al. 2012b), male parental care (Knapp and Kovach 1991), nutritional gifts (Gwynne 1984; Fedorka and Mousseau 2002), hydration (Ivy et al. 1999), replenishment of viable sperm (Ridley 1988; Drnevich et al. 2001), or essential ejaculate-derived compounds (Boggs and Gilbert 1979; Loher et al. 1981; Ursprung et al. 2009). Unlike indirect benefits, obtaining additional direct benefits through multiple mating does not necessarily require mating numerous males (Hunter et al. 1993). Females that repeatedly mate with the same male (i.e. monandry) have the potential to gain as much in fitness as females that mate polyandrously (Bybee et al. 2005; House et al. 2009; *but see* Noble et al. 2013).

Many studies examining direct benefits focus on species in which males provide a nutritious resource during courtship or copulation (i.e. nuptial gift) which females consume and use to increase the number or quality of her offspring (Gwynne 1984; Simmons 1990; Fedorka and Mousseau 2002; Tuni et al. 2013). However, there is evidence that ejaculates comprised only of sperm and accessory fluids can confer significant fitness benefits to females. For example, in some butterflies (*Heliconius helcale, Danaus plexippus, and Heliconius erato)*, nutrients derived from the ejaculate are incorporated into both the production of eggs and female somatic maintenance (Boggs and Gilbert 1979), increasing not only female fecundity but longevity as well.

Field crickets (*Gryllus spp.*) have been a useful model system for examining the fitness advantages of female multiple mating. Males provide an ejaculate comprised only of sperm and accessory fluid and offer no further contribution of resources, protection, or parental care. Empirical evidence for indirect benefits is confounding in *Gryllus*, as studies show that polyandry increases (Tregenza and Wedell 1998; Simmons 2001), decreases (Jennions et al.

2007), or has no effect (Gershman 2010) on offspring performance and hatching success. Alternatively, there is strong evidence demonstrating that mating multiple times increases fecundity regardless of whether females mate monandrously or polyandrously (Simmons 1988; Wagner et al. 2001; Gershman 2010). This suggests that direct benefits obtained from the ejaculate are responsible for the increase in fitness. If ejaculatory substances (e.g. sperm or seminal fluid proteins) are required to stimulate oviposition, then females might remate frequently to replenish these substances. Failing to remate would thus result in decreasing fecundity because as ejaculatory substances decline in quantity through time, they limit female oviposition.

Here, we experimentally examine the fecundity benefit of multiple mating to female *Gryllus texensis* field crickets while also testing whether monandry or polyandry confer greater fecundity. We use an experimental design whereby females are paired with a male either once, twice, or continuously throughout a trial, and twice- and continuously-paired females are allocated either the same male (monandry) or two different males (polyandry) across matings. If females gain fecundity benefits from high mating rates, then we predict a positive relationship between female fecundity and number of mating bouts. Additionally, if the fecundity benefits that females obtain from mating are derived from the ejaculate, then females within each treatment (twice or continuous) will exhibit equal fecundity regardless of whether they mated with one or two males. We also examine whether the act and type (i.e. monandry vs. polyandry) of mating negatively affects female survivorship. We predict that rates of injury and mortality will be highest as females spend more time in the presence of multiple males.

In a separate experiment, we test the hypothesis that multiple mating increases female fitness by causing a higher rate of oviposition throughout the female's lifespan. If ejaculatory substances stimulate oviposition, then the number of eggs that a female lays after mating should decrease through time as the substances are depleted. However, we predict that re-mating will replenish these substances and allow females to lay eggs at a higher rate for longer. Additionally, we test whether multiple mating increases female lifespan, which could increase overall fitness if females that mate more are able to lay more eggs due to living longer.

Materials and Methods

Experimental Animals

Crickets (long-winged *G. texensis*) were originally collected in Austin, TX in 2010 and have been maintained as a laboratory colony for 6−7 generations. Crickets were reared in large communal bins (73 × 41 × 46 cm) until their penultimate instar and were then kept individually in clear plastic 250 ml containers (10 cm diameter × 4.5 cm depth) to ensure virginity. Crickets were housed in an environmentally controlled room maintained at 28 °C on a 12:12 h light:dark cycle. All individuals were supplied cotton-plugged water vials and Special Kitty Premium Cat Food™ *ad libitum*. Experimental crickets were observed daily for eclosion. We standardized cricket age (7-day post-eclosion) and mated status (virgin) for all.

Effect of Number and Type of Mating Bouts on Female Fecundity and Survivorship

We experimentally examined the fecundity benefit and survivorship cost of mating multiple times in female *G. texensis*. We paired females with a male either zero times (i.e.

virgin), once, twice, or continuously throughout a 15-d trial, and 'twice-paired' and 'continuously-paired' females were allocated either with the same male (monandry) or with two different males (polyandry) between mating bouts. At the beginning of the dark period (1200 hours) on Day 0, we transferred each experimental female into a clear plastic mating arena (10 cm diameter × 4.5 cm depth) under a 25-W red light. Females in the virgin treatment remained solitary during this time, but those assigned to mating treatments ('once-paired', 'twice-paired', or 'continuously-paired') were randomly allocated an age-matched virgin male and given 6 h to mate. This method has been proven useful in other studies examining the effect of mating on fitness (Wagner et al. 2001), as most female crickets will mate when given the opportunity. At 1800 hours, we measured female pronotum length to the nearest 0.001 mm (Leica Application Suite V3.8.0, Leica Microsystems. Switzerland), then transferred females to individual oviposition containers (16.5 × 10.5 × 7 cm) supplied with food, water, and moistened ReptiSand (ZooMed, San Luis Obispo, CA, USA) as oviposition substrate. Males were returned to their rearing containers, but those allocated to the 'continuously-paired' treatment were transferred into oviposition containers with their respective female. On Day 8, we again placed females in a mating arena at 1200 hours. Virgin and 'once-paired' females remained solitary while 'twice-paired' and 'continuously-paired' females were provided with either the same male (i.e. monadrous) or with a different male (i.e. polyandrous) from their previous mating bout. At 1800 hours, we transferred females to clean oviposition containers and males that were allocated to the 'continuously-paired' treatment were again housed with their respective females.

On Days 8 and 15 we collected, dried, and sieved oviposition sand, then counted the number of eggs that each female laid. We initially used 574 female crickets; however, 18

monandrous trials were terminated because the male died before the second mating bout. We checked female crickets daily for loss of limbs and mortality throughout the trial. At the end of each trial (Day 15 at 1200 hours) we euthanized females by freezing them at -20 ºC and immediately quantified the number of eggs within their ovaries. Total fecundity for each female was calculated as the sum of all eggs laid and stored in the ovary. Finally, to estimate egg quality, we photographed and measured the length of the five most-posterior (i.e. most developed) eggs in the right ovary.

We later dissected spermathecae of females to determine how many females retained sperm at the end of the 15 d oviposition period. Each spermatheca was transferred to a 1.5 ml microcentrifuge tube containing 75 µl of distilled water, crushed three times using fine forceps, and mixed by vortexing for three 1 s pulses. The entire sample was then spread evenly over a microscope slide, allowed to air-dry, and observed at 400× magnification (Leica DM 2500; Leica Microsystems, Germany) for the presence of sperm.

Female Mating Frequency

In a separate experiment, we determined female mating frequency by observing 36 pairs of crickets throughout a 6-h mating bout to quantify the number of successful copulations completed during that time. Copulation was counted as successful only if a spermatophore was transferred to the female and remained attached for a minimum of 30 minutes. After 6 h, we placed females in oviposition containers to mirror the experimental design used in our previous experiment. One week after the initial mating, we compared mating rates during a second 6-h mating bout to ensure that fecundity differences between monandrous and polyandrous females are not confounded by an increased tendency of

females to mate with familiar or novel mates. Therefore, females were paired with either the same or a different male and observed for 6 h to quantify the number of successful copulations.

Effect of Ejaculate Replenishment on Lifetime Fecundity and Longevity

To determine the effect of ejaculate replenishment on female lifetime fecundity and longevity, we paired male and female crickets in a clear plastic container under a 25-W red light and counted the number of copulations observed during 6 h. Afterward, females were placed alone in individual oviposition containers. One week after the initial mating bout, females were paired with a novel male and again observed for 6 h. Females were then housed in oviposition containers until natural death. During this time we counted the number of eggs laid each week and checked females daily for mortality. Longevity was measured as the number of days a female survived after the first mating bout. To examine the effect of ejaculate replenishment on lifetime fecundity, oviposition rate, and female lifespan, females were separated into three post-hoc treatment groups: 1) mated during the first period but failed to mate during the second (n = 12); 2) failed to mate during the first period but mated during the second (n = 17); or 3) mated during both periods (n = 15). We observed a total of 53 pairs of crickets and females that did not mate during either period were removed from the experiment.

Statistical Analyses

We quantified the effect of mating type (monandry vs. polyandry) on the quantity (total fecundity and number laid) and quality (i.e. size) of eggs of females in the ‘twice-

paired' and 'continuously-paired' treatments. When non-significant, monandrous and polyandrous females were pooled within their respective treatments and the factor was dropped from further analyses. We then investigated the effect of the mating treatment (virgin, 'once-paired', 'twice-paired', or 'continuously-paired') on female fecundity using a generalized linear model (GLM), including female pronotum length as a covariate because cricket fecundity is positively influenced by female body size (Kelly et al. 2014). We compared full to reduced models using AIC, and simplified models by removing interaction terms when appropriate (i.e. homogeneity of slopes). Because our count data (total fecundity and number of eggs laid) suffered from over dispersion and an excess of zeroes, we used 'hurdle' from the 'pscl' package with either a quasipoisson or negative binomial family of errors. These analyses resulted in two-stage data analysis, in which we first determined if treatments differed significantly in the probability of laying eggs (i.e. compared the proportion of individuals that did not lay eggs vs. those that did), and then compared the effect of mating treatment on fecundity for only those individuals that laid eggs. When treatment significantly affected egg quantity, we grouped the data in order to examine treatment differences that were identified *a priori*: virgin versus mated (i.e. pooled 'once-', 'twice-', and 'continuously-paired' females), 'once-paired' versus multiply-paired (i.e. pooled 'twice-' and 'continuously-paired' females), and 'twice-paired' versus 'continuously-paired'. We did this because there is currently no consensus on how to conduct planned contrasts on data analyzed using a two-stage GLM. We controlled for multiple comparisons using the Holm-Bonferroni method to maintain a table-wide type-I error rate of $\alpha = 0.05$ (Holm 1979). To examine the effect of mating treatment and body size (i.e. pronotum length) on egg quality, we analyzed our data using ANCOVA. We first examined the interaction

between mating treatment and pronotum length in order to test the assumption of heterogeneity of slopes. If the interaction term was non-significant, it was removed from the model and an ANCOVA was performed. Sample sizes vary across analyses due to missing data (e.g. some females did not have any eggs in the ovaries). To examine how mating interactions affect female survival, we used a Pearson's chi-squared test to compare the proportion of injured/dead females from each of the mating treatments. Finally, we compared the proportion of females in each treatment that had sperm present/absent from their spermatheca at the end of the 15-day trial using Pearson's chi-squared test.

We examined whether type of mating significantly affects female mating rate using Fisher's Exact test to compare the proportion of monandrous and polyandrous females that successfully copulated during the second 6-h mating bout. Then, because our data were non-normally distributed, we used a Mann-Whitney U Test to determine if mating type affects the total number of ejaculates females accept across all mating bouts.

We examined the effect of multiple mating on female lifespan using a Cox regression (Cox 1972; Fox 2002) to compare the proportion of females surviving after mating for each treatment. We also used a Cox regression to examine the effect of mating treatment on the duration of time a female lays eggs after mating. Because even virgin females lay a baseline number of unfertilized eggs, duration of mating-induced oviposition was determined by the number of weeks a female continued to lay eggs at a rate higher than the average virgin (~10 eggs/week as determined from the first experiment). To compare changes in mating-induced oviposition through time, we used one-sample t-tests to compare the time it took for the mean oviposition rates of females in each treatment to equal that of virgins (i.e. $\leq$10 eggs/week). Next, we examined whether timing of mating and ejaculate replenishment increase the total

number of eggs laid using a GLM with a quasi-Poisson distribution. Our post-hoc mating treatments were included as the main effect of the GLM and duration of mating-induced oviposition was included as a covariate. We compared full and reduced models using AIC with models being simplified by removing interaction terms when appropriate (i.e. homogeneity of slopes). We used treatment contrasts to examine the specific effects of mating early in the reproductive period and ejaculate replenishment on female lifetime fecundity. Finally, we used non-linear regression to model and plot the 2-parameter asymptotic exponential relationship of the cumulative number of eggs laid throughout female lifespan for each treatment. All statistical analyses were performed in R version 2.12 (R Development Core Team 2009) with $\alpha = 0.05$.

Results

Effect of Mating Type on Egg Quantity and Quality

Mating type (i.e. monandry vs. polyandry) did not have a significant effect on the total fecundity of females in the 'twice-' or 'continuously-paired' treatments (GLM: $t = 0.235$; $p = 0.8141$, $df = 316$). Further, mating type had no significant effect on whether females laid eggs (binomial hurdle model: $z = 1.265$; $p = 0.2058$, $n = 317$) or on the total number of eggs laid when they did lay (negative binomial hurdle model: $z = 0.719$; $p = 0.4724$, $n = 302$). Mating type did not significantly affect egg size (ANCOVA: $F = 1.943$; $p = 0.1643$, $df = 3, 309$); however, egg size was positively related to the covariate pronotum length (ANOVA: $F = 7.589$, $p = 0.0062$, $df = 3, 302$). Therefore, females that mated monandrously or polyandrously were pooled within their respective mating treatments for the remaining analyses. See Table 1 for summary statistics of each treatment group.

Effect of Mating Bouts on Egg Quantity and Quality

Mating treatment and female pronotum length had a significant effect on the total fecundity (i.e. the sum of all eggs stored in the ovaries and those that were laid) of a female (Tables 1 and 2). Specifically, multiply-paired ('twice-' and 'continuously-paired') females had significantly higher fecundity than did 'once-paired' females (GLM: $t = 3.562$, $p = 0.0004$, $n = 401$) and larger females had higher fecundity than smaller females (GLM: $t = 7.666$, $p < 0.0001$).

Mating treatment also had a significant effect on whether females were stimulated to oviposit (Tables 1 and 2, Fig. 1a), as mated females were more likely to oviposit relative to virgin females (binomial hurdle model: $z = 6.286$, $p < 0.0001$, $n = 485$). When females were stimulated to oviposit (i.e. lay ≥ 1 egg) the number of eggs that they laid increased significantly with both the number of mating bouts (see Table 2, Fig. 1b) and pronotum length (negative binomial hurdle: $z = 2.576$, $p = 0.0100$, $n = 485$).

Effect of Mating Bouts on Female Survival

The rates of female injury and death significantly differed between mating treatments (Pearson's chi-squared: $\chi^2 = 26.507$, $p < 0.0001$, $df = 3$; Table 1), with risk of injury or death increasing as exposure to males increased. Further, females that mated polyandrously experienced increased rates of injury and death relative to females that mated monandrously in the 'continuously-paired' (Fisher exact test: $p = 0.0096$, $n = 155$) but not 'twice-paired' (Fisher exact test: $p = 0.2223$, $n = 162$) treatment.

Effect of Mating Bouts on Sperm Presence

The proportion of females with sperm present in the spermathecae after 15 days significantly differed between the mating treatments (Pearson's chi-squared: $\chi^2 = 24.200$, $p < 0.0001$, df = 2; Table 1), where a greater proportion of 'twice-' or 'continuously-paired' females had sperm in their spermatheca compared to 'once-paired' females. Further, females that mated polyandrously were more likely to have sperm in the spermatheca relative to females that mated monandrously in the 'twice-paired' (Pearson's chi-squared: $\chi^2 = 7.577$, $p = 0.0059$, df = 1) but not 'continuously-paired' treatment (Pearson's chi-squared Pearson's chi-squared: $\chi^2 = 0.0783$, $p = 0.7796$, df = 1).

Female Mating Frequency

The average number of ejaculates a female received during 6 h was 1.89 ± 0.32 (mean ± se). Females given a new male were not more likely to accept an ejaculate during their second mating period compared to females provided with a familiar male (Fisher's Exact test, $p = 0.7949$, $n = 36$). Monandrous and polyandrous females did not significantly differ in the total number of ejaculates accepted from males (Mann-Whitney U Test: $z = 1.0298$, $p = 0.3030$, $n = 36$).

Effect of Ejaculate Replenishment on Lifespan, Lifetime Fecundity, and Oviposition Rate

We found that multiple mating did not significantly affect female lifespan (Cox regression: $z = 0.659$; $p = 0.5100$, $n = 44$), but did significantly affect the duration of mating-induced oviposition (Fig. 2), where females that mated during both bouts laid eggs for significantly longer than females that mated only during the first (Cox regression: $z = 1.979$;

p = 0.0478, n = 44) or second bout (Cox regression: z = 3.318; p = 0.0009, n = 44). The effect that our mating treatments had on the duration of mating-induced oviposition depended both on how frequently and when (early or late in reproductive life) a female was mated. According to one-sample t-tests, females that mated during both bouts laid eggs at a rate higher than virgins (10 eggs/week) for 4 weeks after the final mating (t = 2.3248, df = 9, p = 0.0451) compared to females that mated only during the first (t = 2.9781, df = 11, p = 0.0126) or second (t = 2.3720, df = 14, p = 0.0326) mating bout that exhibited only 3 weeks of mating-induced oviposition.

Both mating treatment and duration of mating-induced oviposition had significant effects on lifetime fecundity (Figure 3). Specifically, we found that females that mated early in their reproductive life (i.e. only during the first bout) laid significantly more eggs than females that mated later in life (i.e. only during the second bout)(GLM: t = 2.532 p = 0.0154, df = 43). Additionally, ejaculate replenishment significantly increased the total number of eggs laid compared to females that mated only during the first (GLM: t = 3.516, p = 0.0009, df = 43) or the second bout (GLM: t = 3.627, p = 0.0008, df = 43). Across all treatments, duration of mating-induced oviposition significantly increased female lifetime fecundity (GLM: t = 3.101, p = 0.0035, df = 43).

Discussion

As predicted, higher female mating frequencies significantly increased lifetime fecundity independent of whether females mated monandrously or polyandrously. Female *G. texensis* receive only a spermatophore and no other obvious fitness benefits from males (Loher and Dambach 1989), therefore our results clearly demonstrate that female field

crickets gain direct benefits from the ejaculate. Further, because mating frequency did not increase female longevity, the increase in the number of eggs laid by multiply mated females cannot simply be attributed to a longer lifespan. Rather, we found that an increased rate and duration of oviposition was responsible for the greater number of eggs laid by multiply mated females.

Mating resulted in an immediate increase in oviposition rate for all females followed by a significant decline two weeks later, however the total number of eggs laid after mating was dependent on when a female mated. Females that mated on their first encounter with a male (i.e. week 0) laid eggs at a higher rate than females that did not mate until their second encounter (i.e. week 1). Theoretical models show that the consequences of failing to acquire viable sperm in a reasonable amount of time after sexual maturation may considerably reduce an individual's fitness (Kokko and Mappes 2005). Our results demonstrate this empirically, as females that failed to mate with the first available male suffered a significant decline in lifetime reproductive success. Further, these results tentatively support the "trade-up" hypothesis suggested by Jennions and Petrie (2000), where optimally-behaving females should readily mate any male early in the breeding season to ensure fertilization, but then increase their choosiness in subsequent matings to improve the quality or performance of their offspring via indirect genetic benefits. Therefore, females should mate early and often in order to maximize fitness.

We found that mating multiple times prolonged the high rate of oviposition that females experienced directly after mating. When females mated during only a single bout, their oviposition rates immediately and steadily declined each following week until they returned to rates equal to that of virgins during the third week. Females that mated during

both bouts, however, experienced increased rates of oviposition not only after each mating bout, but also for an additional week, providing them with high oviposition rates for four weeks after their final mating. The consequence of this extra week of increased oviposition is that females that mated during multiple bouts laid a greater number of eggs in the first three weeks of their reproductive life than females that mated only once laid during their entire lifespan, demonstrating that the immediate benefits of multiple mating may greatly outweigh the long-term costs that mating poses to female condition and survival. Also, that acquiring ejaculates at multiple points throughout the reproductive period increase both the duration of mating-induced oviposition and the likelihood of sperm presence in the spermatheca supports the hypotheses that ejaculate depletion limits female reproductive success. Mating multiple times is likely a mechanism to replenish sperm and other essential ejaculate-derived compounds (e.g. seminal fluid proteins, prostaglandins, etc.) in order to maintain high rates of oviposition. If mating at higher rates indeed increases female fecundity, why then do so many females choose to not mate when given the opportunity?

Consistent with previous studies, mating had significant costs on female survival. Females that spent more time in the company of males experienced significantly higher rates of injury and mortality, especially those females that mated multiple males. Increased male aggression towards polyandrous females is a common trend, as males often increase mate guarding (Wynn and Vahed 2004; Harts and Kokko 2013) when the risk and intensity of sperm competition is high. In addition to male aggression, female crickets in nature also experience both high rates of predation while searching for mates (Sakaluk and Belwood 1984; Heller and Arlettaz 1994) and high rates of infection from sexually transmitted pathogens (Zuk 1987a,b; Adamo et al. 2014). Because this study was conducted on a captive

cricket population, females were not exposed to either of these threats, therefore the rates of injury and death documented in our study are likely an underestimate of what females experience in the wild, which would likely shift the optimal mating rate.

If female field crickets experience increased rates of injury and mortality from mating with a number of different males, yet doing so does not increase their overall fecundity, then what benefits do they gain by engaging in the high rates of polyandry observed in nature (Bretman and Tregenza 2005)? First, although our results demonstrate that mating once is enough to stimulate oviposition, an increased mating rate resulted in a greater likelihood of sperm presence in the spermatheca, especially in females that mated polyandrously. Therefore, mating multiple males may reduce the likelihood of mating failures or receiving spermatophores void of sperm. Evidence from additional orthopteran species suggest the rates of mating failure is high (Loher and Edson 1973), that males may experience sperm depletion after mating multiple times (Sturm 2011), or that males can strategically decrease the number of sperm that they invest in a given female (Simmons et al. 2007; Thomas and Simmons 2007). Further, our study did not include an investigation of indirect benefits of mating, such as increased offspring quality or performance. Evidence for indirect benefits in field crickets are mixed however, with some studies showing support for the sexy sperm hypothesis (McNamara et al. 2014) or increased hatching success (Tregenza and Wedell 1998; Simmons 2001), and others showing no evidence of increases in hatchability (Jennions et al. 2007; Gershman 2010), offspring performance (Tregenza and Wedell 1998; Simmons 2001; Jennions et al. 2007) or offspring quality (Jennions et al. 2007) after polyandry. Although indirect benefits likely exist and augment female fitness, our study demonstrated that females readily engage in repeated mating with the same male and that the rate of mating

does not differ between females that mate monandrously or polyandrously, suggesting that the majority of benefits that females gain from mating come from substances in the ejaculate, not necessarily indirect benefits.

There are a number of ejaculate components that may be responsible for these direct benefits. First and foremost, females may need to mate frequently to maintain adequate storage of viable sperm. To our knowledge, no one has examined how long after mating sperm remain viable in the spermatheca of crickets. If replenishment of viable sperm is indeed responsible for high mating rates, then we should find that the time at which females remate coincides with the time that the costs of sperm depletion (reduced egg viability) outweigh the costs of mating. However, the fact that females lay a large number of eggs for several weeks after mating, yet will readily remate immediately after a spermatophore is removed suggests that sperm replenishment alone is not responsible for the high rates of mating. Alternatively, seminal fluid proteins play a large role in reproduction in other species (Avila et al. 2011) and may have significant effects in crickets as well. Recently, 21 seminal fluid proteins were identified in the cricket *Teleogryllus oceanicus*, many of which resemble those involved in post-mating changes to female reproduction in other species (Simmons et al. 2012). In *Gryllus*, however, isolated seminal fluid proteins induce only modest short-term oviposition, so although they may play a small role in increasing the fecundity of mated females, it is unlikely that they are the driving factor of increased oviposition or high mating rates (Larson et al. 2012).

One other possibility is that nutrients derived from the ejaculate may be directly responsible for the increased lifetime fecundity of females that mate frequently. Prostaglandin, physiologically active lipid compounds derived from C_{20} polyunsaturated fatty

acids, mediates oviposition behavior in crickets (Loher et al. 1981; Destephano and Brady 1982; Stanley-Samuelson and Loher 1983) and is required to stimulate egg laying after mating. Injecting prostaglandin into females initiates oviposition in a dose-dependent manner, where larger doses stimulate higher rates of oviposition and for a longer period of time than smaller doses (Destephano and Brady 1982; Stanley-Samuelson et al. 1986), similar to what is exhibited by females that receive multiple ejaculates. If the presence of prostaglandin within the spermatheca is ephemeral, ejaculate-derived prostaglandin could provide the underlying reason why females remate frequently even when they have viable sperm remaining in their spermatheca. Whether prostaglandin is provided by males as a way to manipulate female post-mating behavior or is acting as a mating-derived gift is unknown, but that it exacts physiological changes within the female that initiate oviposition is well accepted.

In a comparative context, orthopteran species in which ejaculates are larger also have lower mating rates (Vahed 2006; Jarčuška and Kaňuch 2014), perhaps because females do not need to replenish ejaculates as frequently. Interestingly, mating rates are not correlated with sperm number per se, but are predicted by overall ejaculate size (Vahed 2006), suggesting that non-sperm components have significant effects on female mating rates across species. Because male investment in ejaculate size varies significantly across species (Sturm 2014), a comparative examination of female mating rates, sperm number, and ejaculate composition may provide additional insight into what role sperm, seminal fluid proteins, and accessory fluid components play in the evolution of female mating rates.

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Table 1. Summary statistics (mean ± se) for the effect of number of mating bouts and number of mates (twice and continuously-paired treatments only) on all dependent variables.

Mating treatment	N	Total fecundity	% Laid eggs	# Eggs laid	Egg size (mm)	% Sperm limited	% Injured or dead
Virgin	83	246 ± 12	66%	19 ± 3	2.24 ± 0.01	N/A	7%
Once-paired	85	250 ± 14	91%	92 ± 10	2.26 ± 0.05	39%	3%
Twice-paired	162	311 ± 11	93%	114 ± 9	2.24 ± 0.02	27%	10%
Monandrous	82	334 ± 14	90%	107 ± 13	2.22 ± 0.01	37%	13%
Polyandrous	80	287 ± 16	97%	122 ± 13	2.26 ± 0.04	16%	7%
Continuously-paired	155	279 ± 19	97%	149 ± 10	2.27 ± 0.02	12%	22%
Monandrous	78	264 ± 14	98%	159 ± 14	2.26 ± 0.03	10%	13%
Polyandrous	77	295 ± 11	96%	140 ± 13	2.25 ± 0.01	13%	29%

Table 2. Effect of the number of mating bouts (explanatory factor) and female pronotum (covariate) on total fecundity (ANCOVA), the ability to stimulate oviposition and the number of eggs laid (GLM), and egg size (ANCOVA). *P*-values remaining significant after a Holm-Bonferroni adjustment are bolded. P-values are from contrasts testing for differences between specific treatment comparisons.

Model parameter	*estimate*	*std error*	*test statistic*	*p-value*
Total fecundity				
Virgin vs. mated	0.0571	0.0560	t = 1.020	0.3080
Once- vs. multiply-paired	0.1877	0.0527	t = 3.562	**0.0004**
Twice- vs. continuously-paired	0.0747	0.0445	t = 1.679	0.0942
Pronotum (mm)	0.4904	0.0639	t = 7.666	**0.0001**
Laid vs. Did not lay eggs				
Virgin vs. mated	2.2922	0.3332	z = 6.286	**0.0001**
Once- vs. multiply-paired	0.7428	0.4582	z = 1.621	0.1050
Twice- vs. continuously-paired	1.0301	0.5986	z = 1.721	0.0853
Pronotum (mm)	0.2748	0.5081	z = 0.541	0.5886
Only those females that laid eggs				
Virgin vs. mated	1.7911	0.1796	z = 9.970	**0.0001**
Once- vs. multiply-paired	0.3863	0.1410	z = 2.739	**0.0062**
Twice- vs. continuously-paired	0.3168	0.0102	z = 31.13	**0.0001**
Pronotum (mm)	0.2580	0.0773	z = 2.576	**0.0100**
Egg size (mm)				
Virgin vs. mated	0.0013	0.0270	t = 0.498	0.6184
Once- vs. multiply-paired	0.0016	0.0036	t = 0.671	0.5022
Twice- vs. continuously-paired	0.0085	0.0049	t = 1.691	0.0914
Pronotum (mm)	0.0422	0.0126	t = 3.353	**0.0008**

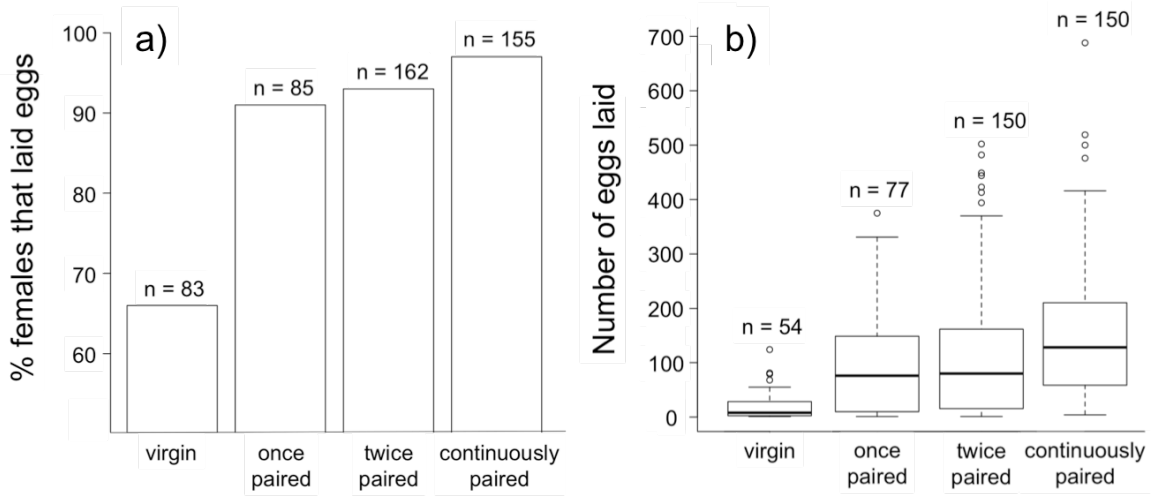

Figure 1. Effect of the number of mating bouts on (a) the ability to stimulate oviposition and (b) the number of eggs laid in the two weeks after mating when oviposition was stimulated. For boxplots, the box represents the lower (25%) and upper (75%) quartiles, the solid dark horizontal line is the median, and the whiskers indicate 1.5 times the interquartile range. Data beyond the end of the whiskers are outliers and plotted as open dots.

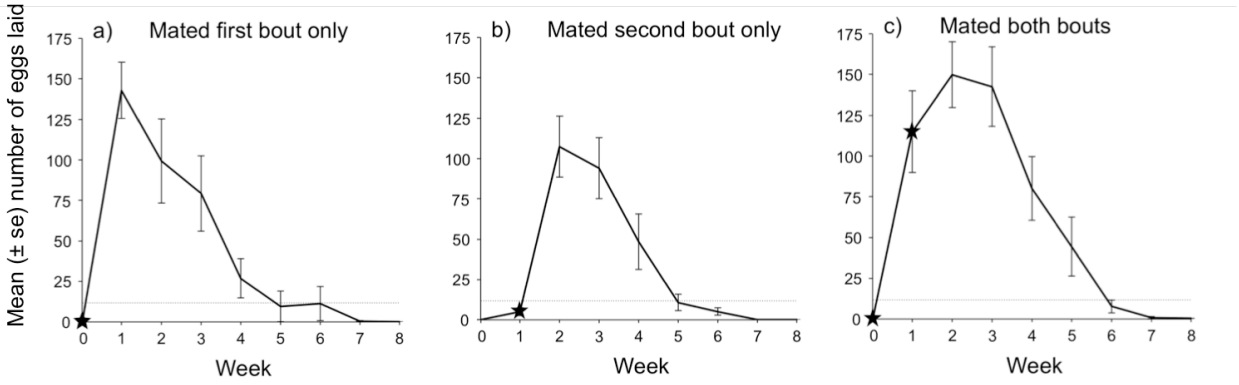

Figure 2. The effect of the number of mating bouts on weekly oviposition rates (mean ± se) in females that mated during (a) the first bout only (n =12), (b) the second bout only (n = 17), or (c) both bouts (n = 15). Stars represent the week(s) that females in each treatment were mated and the horizontal dotted line identifies the average rate of oviposition exhibited by virgin females

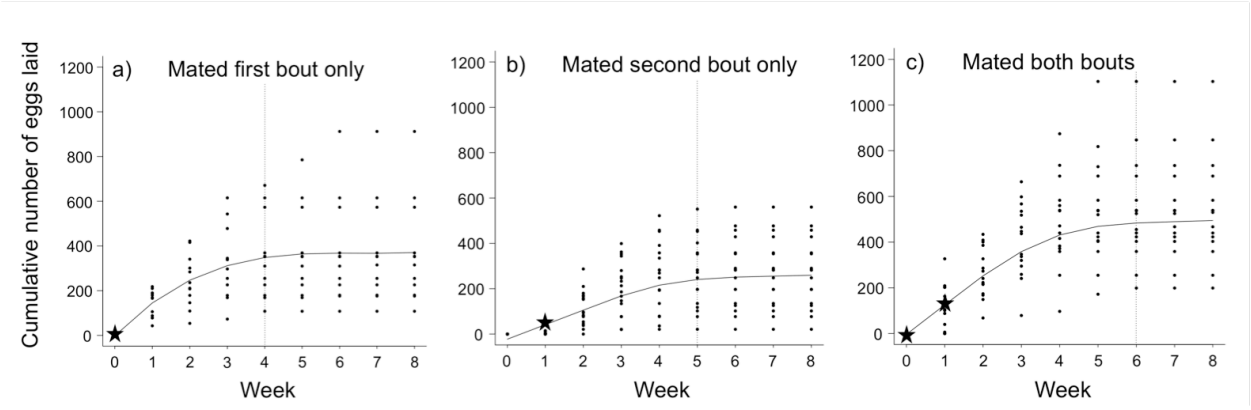

Figure 3. The effect of the number of mating bouts on the cumulative number of eggs laid in females that mated during (a) the first bout only ($y = 384.17(1-e^{-0.53x})$; n = 12), (b) the second bout only ($y = 334.83(1-e^{-0.26x})$; n = 17), or (c) both bouts ($y = 618.30(1-e^{-0.33x})$; n = 15). Individual data points are represented by black dots. Stars represent the week(s) that females in each treatment were mated. The vertical dotted line identifies the point at which oviposition rate is equal to or less than that of virgin females (≤ 10 eggs/week).

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CHAPTER III

MATING FOR MALE-DERIVED PROSTAGLANDIN: A FUNCTIONAL EXPLANATION FOR THE INCREASED FECUNDITY OF MATED FEMALE FIELD CRICKETS?

A paper submitted for publication to a peer-reviewed journal
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Abstract

Direct benefits are considered to be the driving force of high female mating rates, yet species in which females do not receive material resources from males still experience increased fitness from mating frequently. One hypothesis suggests that substances within the ejaculate may boost survival or offspring production. If these materials are limiting to females, they will require continual renewal via mating and could provide a functional understanding of how high mating rates lead to increased female fitness. Using the Texas field cricket, *Gryllus texensis*, we investigated the sexual transfer of prostaglandin E_2, an important mediator of insect reproduction. We determined that like other gryllid species, males include significant quantities of prostaglandin E_2 (PGE_2) and its precursor molecule, arachidonic acid, within the spermatophore. These components are passed to females during copulation and then stored within the spermatheca. We then tested the novel hypothesis that PGE_2 is ephemerally available after mating and that females must frequently mate to maintain access to this limiting compound. We found that PGE_2 within the spermatheca is indeed depleted through time, with only a small amount remaining one week after mating, but that its presence can be maintained at high quantities and for prolonged periods of time by remating. Our results support the hypothesis that high female mating rates increase the amount and availability of

PGE_2 throughout the breeding season, which could explain the positive relationship between female mating rate and fecundity.

Introduction

Direct fitness benefits obtained by females from mating, such as greater fecundity or longevity, have been widely acknowledged as a driving force of the high mating rates observed in nature (Andersson, 1994; Arnqvist and Nilsson, 2000; Reynolds, 1996). Unlike indirect benefits (e.g. genetic diversity of offspring) that often require mating with multiple males, in many cases females can obtain direct benefits regardless of whether they mate repeatedly with the same male or polyandrously with different males. Numerous studies of direct benefits have focused on species in which females receive nutritious food gifts from males that are used to increase reproductive investment after mating (Gwynne, 1984; Simmons, 1990; Vahed, 1998); yet, a positive correlation between offspring production and mating rate is also pervasive in species that receive no obvious material benefits (Dunn et al., 2005; House et al., 2009; Taylor et al., 2008; and reviewed in Arnqvist and Nilsson, 2000; South and Lewis, 2011).

Although fertilization assurance via sperm replenishment is one direct benefit of mating several times (Barbosa et al., 2012; Drnevich et al., 2001; Torok et al., 2003; Uller and Olsson, 2005; Wang and Davis, 2006), many studies have demonstrated the influence that ejaculatory compounds have on reproductive fitness as well. In addition to sperm, ejaculates are composed of a multitude of substances including seminal fluid proteins, sex peptides, salts, sugars, defensive compounds, and lipids (Perry et al., 2013), some of which have profound effects on female fitness (Gioti et al., 2012). For example, seminal fluid proteins in *Drosophila melanogaster* are known to increase egg production (Soller et al., 1999), mediate ovulation and oviposition (Heifetz et al., 2000; Taylor et al., 2008), and elevate sperm storage and utilization (Tram and Wolfner, 1999). Additionally, essential

nutrients in the ejaculate such as salts, sugars, and lipids may act to increase overall female fitness (Boggs, 1990; Gwynne, 2008; Ursprung et al., 2009; Vahed, 1998). If substances comprising the ejaculate significantly increase reproductive output, then females might copulate more frequently to gain access to these fitness-enhancing compounds.

Like *D. melanogaster*, female gryllid crickets do not obtain additional material resources from the male during mating. Despite the lack of nutritional gifts, however, female gryllid crickets that mate multiple times have increased lifetime fecundity relative to females that mate only once (Simmons, 1988; Wagner et al., 2001) and egg output is positively correlated with the number of ejaculates received (Gershman, 2007; Gershman, 2010). Indirect benefits do affect hatching success (Tregenza and Wedell, 1998) and offspring quality (Simmons, 1988) and should therefore not be overlooked; however, a functional explanation of how females receive direct benefits when only an ejaculate is acquired remains an outstanding question.

That mating multiple times results in increased in fecundity in gryllid crickets might be a function of prostaglandins contained within the spermatophore. Prostaglandins are oxygenated metabolites of C_{20} polyunsaturated fatty acids that are fundamental to many aspects of animal physiology (Craig, 1975; Harris et al. 2002; Wolfe and Coceani, 1979) and are found in the ejaculates of various animals (Kennedy et al., 2003; Kobayashi et al., 2013; Loher et al. 1981; Templeton et al., 1978). The biological significance of prostaglandins in mediating physiological actions essential for reproduction is well understood in mammals (Didolkar and Roychowdhury, 1980; Herrero et al. 1997; Kurzrok and Lieb, 1930; Marey et al., 2013), and is considered to be important in invertebrate reproduction as well. Prostaglandins are found in cricket seminal fluid (Loher et al. 1981) and have noted effects

on the reproductive physiology of two species, *Teleogryllus commodus* and *Acheta domesticus*, in mediating egg production and oviposition in mated females (Loher and Edson, 1973; Murtaugh and Denlinger, 1985). Changes in reproductive behavior as a consequence of mating are similar to the effects exhibited by females that have been experimentally treated with prostaglandin. Injection of prostaglandin into virgin females induces oviposition in a dose-dependent fashion (Destephano and Brady, 1977; Loher, 1979; Loher et al., 1981; Tanaka, 2014), increasing both the rate (Destephano and Brady, 1977) and duration (Destephano et al., 1982) of egg laying. Specifically, prostaglandin E_2 (PGE_2) is successful at effecting these physiological changes (Stanley-Samuelson et al., 1986a) by stimulating nerves located within the female genital chamber (Sugawara and Loher, 1986).

Seminal fluids from *A. domesticus* and *T. commodus* contain approximately 20 pg of preformed PGE_2 (Loher et al., 1981) that originates from the testes (Murtaugh and Denlinger, 1982). Male crickets pass this PGE_2 to females with the remainder of the ejaculate (testicular and accessory gland material) in a small package called a spermatophore. During copulation, the spermatophore is placed into the female's genitalia so that sperm and seminal fluids can be transferred to and stored in the female's internal sperm storage organ, the spermatheca. Although PGE_2 is absent in the spermathecae of virgin females, mated females have an average of 500 pg of PGE_2 (Loher et al., 1981; Stanley-Samuelson and Loher, 1983), far more PGE_2 than contained in a single spermatophore. This suggests that not only is preformed PGE_2 transferred to females at the time of mating, but that females also synthesize additional PGE_2 after copulation. Post-copulatory synthesis of PGE_2 is possible because males also transfer the precursors of PGE_2 in the seminal fluid (Destephano et al., 1974; Loher et al., 1981; Stanley-Samuelson et al., 1987), which include the enzyme complex

prostaglandin synthetase derived from the testes (Tobe and Loher, 1983) and the substrate arachidonic acid (20:4n−6) derived from both the testes and accessory glands. Stanley-Samuelson et al. (1987) traced the transfer of radioactive arachidonic acid from males to females and demonstrated that females indeed use the substrate obtained from mating to synthesize additional PGE_2 post-copula. Interestingly, most insects must obtain arachidonic acid from the diet since few are capable of synthesizing it *de novo* (Blomquist et al., 1991), however some crickets retain this ability. Male *T. commodus*, for example, can synthesize arachidonic acid (Jurenka et al., 1988) as well as prostaglandins (Stanley-Samuelson et al., 1986b) *de novo* within the reproductive organs, thereby providing males a non-dietary source of these essential compounds. Because prostaglandin is quickly metabolized in the hemolymph and excreted from the body (Stanley-Samuelson and Loher, 1985), its storage in the spermatheca is relatively ephemeral (Destephano and Brady, 1977) and females likely need to mate frequently to maintain their supply of PGE_2.

Our current understanding of the reproductive effects of prostaglandin in crickets is derived from two species, *T. commodus* and *A. domesticus*. Here, we extend our knowledge by investigating the sexual transfer of PGE_2 and its precursor arachidonic acid in the Texas field cricket, *Gryllus texensis*, a species that has not yet been used to study the reproductive effects of prostaglandin. *Gryllus spp.* are frequently used in studies demonstrating the benefits of mating, where females that mate multiple times indeed have increased lifetime fecundity relative to once-mated females (Gershman, 2010; Wagner et al., 2001; Worthington and Kelly, unpublished), but females must remate throughout the breeding season to obtain this reproductive benefit as mating multiple times in a single bout does not increase fecundity (Simmons, 1988; Worthington and Kelly, unpublished). We hypothesize

that the functional mechanism underlying the positive relationship between mating rate and fecundity in *G. texensis* is the prostaglandin received from the male, such that mating more frequently increases the amount of prostaglandin available to females, and mating throughout the breeding season allows females to replenish this important and ephemeral egg-laying stimulator.

Our goal in this paper is to provide a detailed explanation of the manufacture and transfer of PGE_2 by males and its use by females in *G. texensis*. From the male perspective, we first test the hypothesis that like other gryllids, male *G. texensis* package PGE_2 into the spermatophore. We accomplish this by directly measuring the quantity of PGE_2 and arachidonic acid in individual spermatophores. Second, we examine the origin of the preformed PGE_2 that is in the spermatophore. We predict that PGE_2 is manufactured in the testes and test this prediction by measuring the amount of PGE_2 in the spermatophores of males that have had their testes experimentally removed (i.e. castrated). If the preformed PGE_2 within the seminal fluid is indeed derived from the testes and is responsible for the physiological changes we see in females after mating, then PGE_2 will be absent from the spermatophores of castrated males and these spermatophore contents will not stimulate oviposition in females.

From the female perspective, we test the hypothesis that male-derived PGE_2 is transferred to females during mating and that mating more frequently increases the amount of PGE_2 acquired. We test this hypothesis by comparing the quantity of PGE_2 in the spermathecae of virgin and mated females. We predict that virgin females will have negligible amounts of PGE_2 in the spermatheca, that spermatheca from once-mated females will contain the same quantity of PGE_2 as is present in a single spermatophore, and that

females mated four times will have significantly more PGE_2 than both virgin and once-mated females. We then test whether females synthesize additional PGE_2 after mating by analyzing spermathecae immediately or 24 hours after copulation. We predict that the quantity of PGE_2 within the spermatheca will increase within 24 hours after copulation due to female synthesis of the compound, thereby exceeding the amount of PGE_2 that can be found within a single spermatophore. Next, we test the hypothesis that PGE_2 found within the spermatheca is only ephemerally available by measuring its quantity 3, 24, and 168 hours after copulation. We predict that after the initial increase at 24 h, the quantity of PGE_2 will decrease as the time since last mating increases. Finally, whether frequent mating throughout the breeding season increases the quantity of PGE_2 available to the female is unknown. We test this novel hypothesis by comparing the quantity of PGE_2 in the spermathecae of females that mated with a male once or *ad libitum* and were then allowed to oviposit for 168 h. We predict that the spermathecae of females mated *ad libitum* will have more PGE_2 relative to the spermathecae of females that mated only once.

According to previous studies, the prostaglandin that males transfer to females during copulation initiates oviposition and greater quantities of prostaglandin (via injections) increase the number of eggs a female lays; therefore, if females can accumulate and maintain continual access to mating-derived PGE_2 by mating multiple times, then prostaglandins may provide a functional mechanism linking higher female mating rates with increased fecundity.

Materials and Methods

Experimental Animals

Crickets (long-winged *G. texensis*) were lab-reared descendants of individuals originally collected in Austin, TX (USA) in the fall of 2010, 2012, and 2013. The laboratory colony of crickets was reared in large communal bins (73 × 41 × 46 cm) until the penultimate instar, after which crickets were kept individually in clear plastic 250 ml containers (10 cm diameter × 4.5 cm depth) until sexual maturity. All crickets were housed in an environmentally-controlled room (27 °C, 12:12 h light:dark cycle) and were supplied with cotton-plugged water vials and dry cat food (Special Kitty Premium Cat Food^{TM}) *ad libitum*. We checked the crickets daily for eclosion. Once the cuticle of a newly eclosed cricket had hardened (later that same day), we measured its pronotum length (the distance between the anterior and posterior edges of the pronotum) to the nearest 0.001 mm using a stereomicroscope (Leica S6D, Leica Microsystems, Germany) equipped with Leica LAS image analysis software (Leica Application Suite V3.8.0, Leica Microsystems. Switzerland). Body mass was recorded to the nearest 0.01 mg on an electronic scale (Denver Instruments TP-64).

Arachidonic acid analysis

Arachidonic acid levels in individual spermatophores and spermathecae were analyzed as methyl esters using the procedure described by Choi et al. (2002). Samples were extracted overnight with 50 µl chloroform/methanol (2/1) containing triheptadecanoin as an internal standard. Methyl esters were made by base methanolysis and analyzed by gas chromatography-mass spectrometry (GC/MS) using a Hewlett-Packard 5890 II mass

selective detector coupled with a Hewlett-Packard 5890 GC equipped with a Carbowax (Alltech, Deerfield, IL) capillary column (30 m x 0.25 mm). The oven temperature was programmed at 60°C for 1 min, then 10°C/min to 230°C and held for 10 min.

PGE_2 analysis

To confirm that the prostaglandin present in spermatophores was PGE_2, we used a fluorometric high-pressure liquid chromatography (HPLC) procedure previously described by Jurenka et al. (1999). Briefly, we pulverized 36 spermatophores in 500 µl ethyl acetate, centrifuged them at 10,000 rpm for 2 min, and removed the supernatant. We repeated this three times and then dried the combined extracts under nitrogen. The combined extracts were reconstituted in 4 µl ethyl acetate and derivatized by adding 1 µl of a 9-anthryldiazomethane (Setareh Biotech, Eugene, OR) solution (5µg/µl in ethyl acetate) and incubating in the dark at room temperature for 4 h. After 4 h, the sample was diluted with ethyl acetate and injected onto an HPLC silica normal-phase column (Whatman Partisphere Silica, 4.6 x 125 mm) using a Beckman System Gold solvent delivery system (Fullerton, CA) equipped with a Hewlett-Packard 1046A fluorescent detector (Wilmington, DE) with excitation and emission wavelengths of 250 and 410 nm, respectively. We used a gradient starting at 100% hexane to 20% 2-propanol in 20 min at a flow rate of 1 ml/min to separate and check for the presence of specific prostaglandins.

We measured the amount of PGE_2 in each sample (spermatophore or spermatheca) using a PGE_2 specific EIA according to manufacturer's instructions (Amersham Prostaglandin E_2 Biotrak EIA System, GE Healthcare). Samples maintained at −80 °C were pulverized using sterile pestles in 120 µl of lysis reagent 1 from the assay kit and then

centrifuged at 10,000 rpm for 1 min. Supernatants were analyzed in duplicate in a 96-well assay plate. Serially diluted PGE_2 standards were also run in duplicate starting at 320 pg down to 1.25 pg. We used GraphPad Prism 4 (GraphPad Software 2005) to calculate the concentration of PGE_2 within each well of the assay plate. The minimum level of detection was about 1.25 pg PGE_2 per sample. We then calculated the total amount of PGE_2 in each spermatophore or spermatheca based on its initial dilution in lysis reagent 1.

Spermatophore sample collection and analysis

Spermatophores used in some of our analyses were collected from mated females directly after copulation. Copulation consists of the male transferring a spermatophore to the mounted female and securing it in place with an attachment plate inserted above the female's subgenital plate. Then, the male threads the spermatophore through the female's genital opening and into the spermathecal duct. The ejaculate is then transferred and the spermatophore capsule is retained externally (Loher and Dambach, 1989). Using fine forceps, we collected each spermatophore by carefully dislodging the attachment plate from the female immediately post-copula before any of its contents could be transferred. All spermatophores were stored in 1.5 mL centrifuge tubes at −80 ºC until the day until AA and PGE_2 contents were quantified.

To test our first hypothesis that *G. texensis* package prostaglandin and its precursors into ejaculates, we analyzed individual spermatophores (n = 18) using GC/MS to verify the presence and quantity of the prostaglandin precursor AA. Additionally, we collectively analyzed 36 spermatophores using HPLC to verify the presence of PGE_2 within spermatophores, and analyzed individual spermatophores (n = 23) using a PGE_2-EIA to

quantify the amount of PGE_2 present within a single spermatophore. Because PGE_2 is also required by males to achieve a number of physiological tasks, we recorded the pronotum width and mass for each male to test whether male size affects the quantity of PGE_2 within the spermatophore.

Spermatophores for additional analyses were collected directly from males by pulling down the subgenital plate and gently palpating the abdomen (Kerr et al., 2010). This method was used to standardize the collection time between males. The formation of the spermatophore prior to courtship has been intricately described for *G. bimaculatus* (Hall et al., 2000), yet there has been no research specifically indicating when prostaglandins are incorporated in the spermatophore. To ensure that collection method did not affect PGE_2 presence and quantity, we collected two spermatophores from thirteen mature males, one of which was collected off of the female after mating and the other was collected directly from the male using the method described above. We then analyzed the quantity of PGE_2 in each of the spermatophores using a PGE_2-EIA.

To determine if males consistently package the same quantity of PGE_2 into each of their spermatophores and whether this quantity varied between males, we analyzed two consecutive spermatophores from randomly-selected mature males (n = 18). Male crickets form and then store a spermatophore for 2-3 days before ejecting it to form a fresh one (Reinhardt and Siva-Jothy, 2005). Because the stability and longevity of PGE_2 within the spermatophore is unknown, we standardized the age of spermatophores by collecting each directly from the male at 1300 hours for three consecutive days. The first spermatophore collected was discarded due to its unknown time of formation, and the quantity of PGE_2 in each of the remaining two spermatophores was analyzed using a PGE_2-EIA.

To test the hypothesis that ejaculate-derived PGE_2 originates in the male gonads, we surgically removed the testes from males (n = 13) and compared the quantity of PGE_2 in the spermatophores with those of sham-castrated males (n = 5). We castrated males following the procedure detailed in Larson et al. (2012). Briefly, males 5-6 days post-eclosion were randomly divided into two groups: 1) both testes removed, or 2) sham-castrated to control for the effects that surgery may have on spermatophore PGE_2 content. Prior to surgery, males were cold-anesthetized for 4.5 min in a −20 ºC freezer. A lateral incision was made through the dorsal intersegmental membrane between the 2nd and 3rd abdominal segments. For castrated males, fine forceps were used to completely remove each testis, whereas sham-castrated had their body cavity probed in a similar manner but without the removal of any organs. We then used Vetbond^{TM} Tissue Adhesive (3 M, St. Paul, MN, USA) to seal the incision, and placed each male in a plastic container with a water vial, an egg carton shelter, and a piece of cat food. After 2 days recovery, males were paired daily with multiple receptive females and allowed to mate in order to deplete the testes-derived components stored in the seminal vesicles. We regularly examined spermatophores from each male for sperm under a compound light microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany). If sperm were observed, males were provided with virgin females and allowed to continue mating for another 24 hours. If no sperm were observed, we considered the males depleted of testes-derived components and ready for sample collection. Sham-castrated males were also allowed to mate with receptive females for 5 days before samples were collected. After mating and sperm-depletion, males were again isolated and two spermatophores were collected from each male 24-hours apart. Again, only the second spermatophore was analyzed for PGE_2 quantity using a PGE_2-EIA.

To test the hypothesis that females require an intact ejaculate containing tested-derived substances (i.e. PGE_2 and sperm) to stimulate egg laying, we randomly assigned 10-d old, sexually mature female crickets to one of three treatments where they 1) remained virgin, 2) were paired with a castrated male that provided an ejaculate comprised of only accessory fluids, or 3) paired with an sham-castrated male that provided an ejaculate comprised of both accessory gland and testes-derived substances. Females were transferred to a clear plastic mating arena (10 cm diameter × 4.5 cm depth) and placed in a dark room under a 25-W red light. Virgin females (n = 25) were paired with a male but were prevented from mating by gently shaking the container. Females assigned to the other treatments were paired with a single un-manipulated (n = 24) or castrated male (n = 20) and allowed to mate. Once a spermatophore was successfully transferred, the male was removed from the arena and females were monitored to ensure that the spermatophore remained attached for 45 min before it we manually detached it. All females were then transferred to individual containers (16.5 × 10.5 × 7 cm) and supplied with one water vial, two pieces of dry cat food, and a 2-oz plastic dish (3 cm diameter × 2 cm depth) of moistened ReptiSand (ZooMed, San Luis Obispo, CA, USA) for oviposition substrate. After three days, we dried the sand at room temperature for 24 hours and quantified the number of eggs laid by each female.

Spermatheca sample collection and analysis

Spermathecae from virgin and mated females were dissected out of cold-anesthetized individuals through a longitudinal incision in the ventral abdomen. The failure to transfer an ejaculate during copulation frequently occurs in virgins (Loher and Edson, 1973); therefore, we examined the spermatheca of mated females to ensure they contained an ejaculate. This

was done visually, as spermathecae from females that have not received an ejaculate are small, flaccid, and clear; whereas those from females that have received an ejaculate are large, turgid, and opaque. All spermathecae were stored individually in 1.5 mL centrifuge tubes at −80 ºC. Spermatheca from virgin (n = 8) and mated (n = 4) females were individually analyzed using GC/MS to measure the quantity of arachidonic acid present.

To establish that PGE_2 is passed to females during copulation and to test the hypothesis that mating multiple times increases the quantity of PGE_2 found in females, the spermathecae of females mated zero (n = 14), one (n = 11), or four times (n = 6) were analyzed. Females 10-14 d post-eclosion were randomly assigned to a treatment group, and females in the mated treatments were paired with sexually-mature virgin males. For females mated four times, a novel male was used for each mating. Spermathecae were dissected and frozen within 3 h of the first copulation, and were later analyzed for PGE_2 quantity using a PGE_2-EIA.

To test the hypotheses that females synthesize additional PGE_2 after copulation and that the PGE_2 stored in the spermatheca is only ephemerally available, we compared the amount of PGE_2 present in the spermathecae of females 3 h (n = 11), 24 h (n = 47), and 168 h (n = 30) post-copula. Females were allowed to copulate with a single virgin male and spermatophores were removed after 45 min of attachment. After spermatophore removal, females were transferred into individual containers and supplied with water vials. After 3 h, 24 h, and 168 h, spermathecae from females in the respective groups were dissected and later analyzed for PGE_2 quantity using a PGE_2-EIA.

Finally, to test the hypothesis that repeated mating over time prolongs the presence and maintains the quantity of PGE_2 present in the spermatheca, we analyzed the quantity of

PGE_2 in the spermathecae of females assigned to one of two treatment groups: 1) mated once and the spermatheca dissected after 168 h (n = 9), or 2) reared in the presence of multiple males (i.e. given the opportunity to mate continuously throughout the trial) and the spermatheca dissected after 168 h (n = 9). Again, spermatophores were removed from mated females after 45 min of attachment. Females were maintained in individual containers supplied with a water vial and a piece of cat food until the day of dissection. All spermathecae were analyzed for PGE_2 quantity using a PGE_2-EIA.

Statistics

We used Pearson correlations to examine the relationship between male size (i.e. mass and pronotum length) and the amount of PGE_2 present in spermatophores. We used a paired t-test to examine the effect of collection method, either pre- or post copula, on the amount of PGE_2 detected in each of a male's spermatophores (log-transformed). To examine whether males consistently package the same amount of PGE_2 into each of their spermatophores, we ran a one-way repeated measures ANOVA where male identity was entered as a random factor and spermatophore (first or second) was the main treatment factor. We also used a paired t-test to determine if the order of spermatophore collection had a significant effect on PGE_2 quantity. We used a nonparametric Mann-Whitney test to determine whether castrated males had significantly different quantities of PGE_2 (log-transformed) present in their spermatophores relative to sham-castrated males. Last, we investigated the effect of accessory fluids and testes-derived substances on the number of eggs laid by focal females using general linear modeling (GLM) with a Poisson family distribution while statistically controlling for body size.

For females, we used an ANOVA to examine the effect of mating and time since last copulation on the amount of PGE_2 detected in the spermathecae (log-transformed). First, we examined how mating either zero, one, or four times affects the quantity of PGE_2 stored in the spermatheca to determine if there is a positive correlation between PGE_2 and mating rate. Next, we investigated whether additional PGE_2 was synthesized after mating and how long it remains by comparing the amount of PGE_2 in the spermathecae of once-mated females 3, 24, and 168 hours post-copula. Last, using a one-tailed *t*-test, we examined whether mating multiple times throughout the 168-h trial period increases the availability of PGE_2 relative to females that mated only once at the beginning of the trial.

We checked that our data met model assumptions by examining the residuals for normality and homogeneity of variances. Summary statistics are reported as mean ± SD. All statistical analyses were performed in R version 2.12 (R Development Core Team 2009) with $\alpha = 0.05$.

Results

Arachidonic acid and prostaglandin in spermatophores

The GC/MS results indicated that spermatophores contain 43 ± 19 ng of arachidonic acid (AA, 20:4n−6) (Fig. 1a), in addition to other common fatty acids. Based on retention time and mass spectra comparison to known standards (Christie, 2014), we found 156 ± 87 ng of 5,11,14-20:3, a structural analog of AA. The importance of this analog in the seminal fluid has yet to be determined, but apparently it cannot be converted into PGE_2 (Berger et al., 2002). The fluorometric-HPLC results confirmed the presence of prostaglandin within the spermatophore and identified the most common form as PGE_2. The PGE_2-EIA detected an

average of 17.2 ± 14.3 pg of PGE_2/spermatophore (Fig. 1b). There was no correlation between the amount of PGE_2 detected in spermatophores and male pronotum width (Pearson correlation: $r = 0.1776$, $t = 0.8268$, df = 21, $P = 0.4176$) or mass (Pearson correlation: $r = 0.0421$, $t = 0.1931$, df = 21, $P = 0.8487$).

The quantity of PGE_2 in spermatophores collected from females immediately after copulation (15.0 ± 20.6 pg) did not significantly differ from spermatophores collected directly from males pre-copula (23.6 ± 36.3 pg) (two-sample t-test: $t = 0.5703$, df = 12, $P = 0.5790$). Additionally, males did not consistently package the same amount of PGE_2 in sequential spermatophores that they formed (one-way repeated measures ANOVA: $F = 1.534$; df = 1, 17; $P = 0.1917$) and spermatophore collection order did not affect the average quantity of PGE_2 contained in subsequent spermatophores (1st spermatophore = 20.7 ± 18.7 pg; 2nd spermatophore = 19.6 ± 12.5 pg) (paired t-test: $t = -0.2966$; df = 17; $P = 0.7704$).

Effect of castration on PGE_2 quantity in spermatophores and post-mating oviposition in females

Castrated males (7.5 ± 4.3 pg) had significantly less PGE_2 present in their spermatophores than sham-castrated males (185 ± 110.4 pg) (Mann-Whitney test: $W = 0.00$; df = 18; $P = 0.0004$). We found a significant effect of mating treatment on the number of eggs laid in the three days after mating. Females that mated with sham-castrated males laid significantly more eggs than virgins ($z = 24.212$; df = 49; $p < 0.0001$) or females that mated with castrated males ($z = 14.849$; df = 44; $p < 0.0001$) (mean number of eggs laid ± SE: virgin 2.5 ± 1.8; castrated 0.5 ± 0.2; sham-castrated 58.9 ± 12.6).

Analysis of arachidonic acid in spermathecae

The GC/MS results indicated that spermathecae from virgin females contained only 6 ± 4 ng of AA compared to the 59 ± 15 ng present in the spermatheca of mated females. The level of 5,11,14-20:3 was about 3 times as much as AA in both virgin and mated spermathecae (17 ± 14 ng, 139 ± 65 ng respectively). This structural analog of AA was also found in large amounts in the spermatophore (see above), but its importance in cricket reproduction is unknown.

Transfer and accumulation of PGE_2 in spermathecae via copulation

Females that mated once tended to have more PGE_2 (15.9 ± 22.5 pg) than virgin females (4.4 ± 5.6 pg), but this difference was only approaching significance ($t = -1.823$; df = 24; $P = 0.0789$) (Fig. 2a). Additionally, females that mated four times had significantly more PGE_2 (191.1 ± 137.9 pg) than once-mated females ($t = 4.254$; df = 16; $P = 0.0002$).

Post-copula synthesis and presence of PGE_2 in the spermathecae

Spermathecae (Fig. 2b) that were dissected 24 h after copulation (269.9 ± 248.7 pg, n = 47) contained significantly more PGE_2 than spermathecae that were dissected only 3 h after copulation (15.9 ± 22.5 pg, n = 11) (Fig. 2b; $t = 9.00$; df = 57; $P < 0.0001$). Alternatively, spermathecae dissected 168 h after copulation (13.5 ± 6.5 pg, n = 30) had significantly less PGE_2 than females dissected after 24 h ($t = 14.17$; df = 76; $P < 0.0001$). As predicted, females that were allowed to mate *ad libitum* throughout the trial period had significantly more PGE_2 in the spermatheca after 168 h (645.0 ± 204.8 pg) than females that were allowed to mate only once (25.4 ± 14.1; Fig 2c; $t = 11.411$; df = 17; $P < 0.0001$).

Discussion

We found that in *G. texensis*, PGE_2 and its precursor AA are present in the spermatophore, passed to females during copulation, and stored then within the spermatheca. Our results also indicate that females synthesize additional PGE_2 within 24 hours after mating. This is most likely achieved by females converting the substrate acquired from the accessory fluid into PGE_2, as has been demonstrated in a previous study that used radioactive AA to trace the fate of male-derived AA in *T. commodus* (Stanley-Samuelson et al., 1987). Further, our results are the first to demonstrate that although PGE_2 is depleted through time, its presence can be maintained at high quantities for prolonged periods of time by mating frequently. Our results show that females acquire PGE_2 via mating and support the hypothesis that high mating rates increase the amount and availability of PGE_2 throughout the breeding season.

That fecundity increases with the number of copulations could be due to a greater quantity of accumulated PGE_2. Previous studies have shown that prostaglandin mediates oviposition in crickets (Loher, 1979; Loher et al., 1981) with larger doses increasing the number of eggs a female lays (Destephano and Brady, 1977; Destephano et al., 1982; Tanaka, 2014). We also know that mating more frequently increases the number of eggs females produce and lay (Gershman, 2007; Gershman, 2010; Loher and Edson, 1973; Murtaugh and Denlinger, 1985) even though a single mating can often provide enough sperm to fertilize all of a females' eggs (Murtaugh and Denlinger, 1985; Simmons, 1988). In our study, we predicted that females that mated at higher rates would have increased quantities of PGE_2 relative to females that mated fewer times, and that only females that mated to males that provided PGE_2 would lay eggs. Our results supported this hypothesis and confirmed that

accessory fluids alone do not induce oviposition, but that female fecundity increases only after receiving a spermatophore containing preformed prostaglandin. However, because both sperm and PGE_2 are derived from the testes, separating the effects of these two factors on female reproductive physiology poses a challenge.

Previous studies on gryllids have used irradiation techniques to kill sperm or prevent spermatogenesis (Murtaugh and Denlinger, 1980; Murtaugh and Denlinger, 1987), or alternatively, have employed a number of prostaglandin injections or prostaglandin synthesis inhibitors to more directly alter *in vitro* prostaglandin concentrations (Murtaugh and Denlinger, 1980; Destephano and Brady, 1977; Murtaugh and Denlinger, 1982). The limited results using these techniques have been mixed and difficult to reproduce. Mating females to irradiated males has led to either normal (Backus, 1986) or decreased (Murtaugh and Denlinger, 1980) rates of oviposition relative to females mated to unmanipulated males. There have been similarly mixed results with the effects of prostaglandin synthesis inhibitors on oviposition (Destephano and Brady, 1977; Murtaugh and Denlinger, 1980), although direct injection of PGE_2 into females consistently results in increased egg laying (Destephano and Brady, 1977; Loher et al., 1981; Stanley-Samuelson et al., 1986a; Tanaka, 2014). Once refined, these techniques will likely be essential in isolating the effects that PGE_2 and sperm have on female reproductive success.

Although previous studies support that ejaculate-derived prostaglandin plays a major role in mediating oviposition behavior, additional reproductive functions of prostaglandins have been proposed. For example, ovarian follicle development and maturation depend on prostaglandin signaling in *D. melanogaster* (Tootle and Spradling, 2008) and the silkmoth, *Bombyx mori* (Machado et. al, 2007), and eggshell production is coordinated by

prostaglandins in *D. melanogaster* (Tootle et al., 2011). Whether exogenous PGE_2 obtained via mating affects ovarian follicle development and maturation in crickets is unknown, but research has shown that high female mating rates increase not only the rate at which females lay eggs, but also induces egg production as well (Loher, 1979; Worthington and Kelly, unpublished), suggesting that ejaculate-derived PGE_2 has a positive effect on both egg development and oviposition. Alternatively, from the male perspective prostaglandins play various roles in sperm maintenance in mammals, including sperm motility (Didolkar and Roychowdhury, 1980), viability (Hayashi et al., 1988), protection from phagocytosis (Marey et al., 2013), and enhancing the acrosome reaction necessary for the fusion of sperm and egg (Herrero et al. 1997). To our knowledge, there are no studies examining the importance of prostaglandin on sperm maintenance in insects, so further research will be required to understand these effects.

Finally, theory suggests that male fitness increases with mating rate, while one or few matings are sufficient for females to gain maximum reproductive success (Bateman, 1948). Empirical research suggests otherwise, as females gain reproductive fitness by mating at high rates despite the costs it has on survival (South and Lewis, 2011). These positive effects of mating multiple times are often the result of ejaculate-derived substances provided by the male, yet whether these substances are a form of a nuptial gift or are manipulative substances that evolved out of sexual conflict remains to be seen. Accessory substances in the seminal fluid are sometimes responsible for decreases in female survival (Green and Tregenza, 2009) and increases in female refractory behavior (Gwynne 1986, Torres-Vila et al. 1997), thereby providing some benefit to the male at a cost to female fitness. Because PGE_2 is required for many essential physiological functions, however, it could provide females with alternative

benefits aside from increased fecundity. For example, PGE_2 is a major mediator of the insect immune system (Stanley and Kim, 2014). If females utilize ejaculate-derived PGE_2 to initiate an immune response after mating, then females could gain a significant fitness advantage in fighting off the many pathogens that they face during the breeding season (Knell and Webberley, 2004). In fact, mated female crickets have increased survival after exposure to a bacterial pathogen relative to virgin females (Shoemaker et al., 2006), therefore it is possible that the functional mechanism for this heightened post-mating immunity is ejaculate-derived PGE_2.

In conclusion, we found that *G. texensis* males provide both AA and PGE_2 in their spermatophores and that these are transferred to the female spermathecae after copulation. Although there is a limited amount of preformed PGE_2 passed immediately after mating, females use the AA and enzyme complex prostaglandin synthetase obtained from males to synthesize more PGE_2 within 24 hours of copulation. PGE_2 is found in the spermatheca for extended periods of time; however, that it decreases in quantity as time-since-last-mating increases suggests it is ephemeral. Most importantly, female *G. texensis* crickets that mate multiple times throughout their lives can maintain continual access to PGE_2. We hypothesize that the continual need for PGE_2 is (at least partially) responsible for the high mating rates observed in crickets and could explain the positive relationship between fecundity and mating rate.

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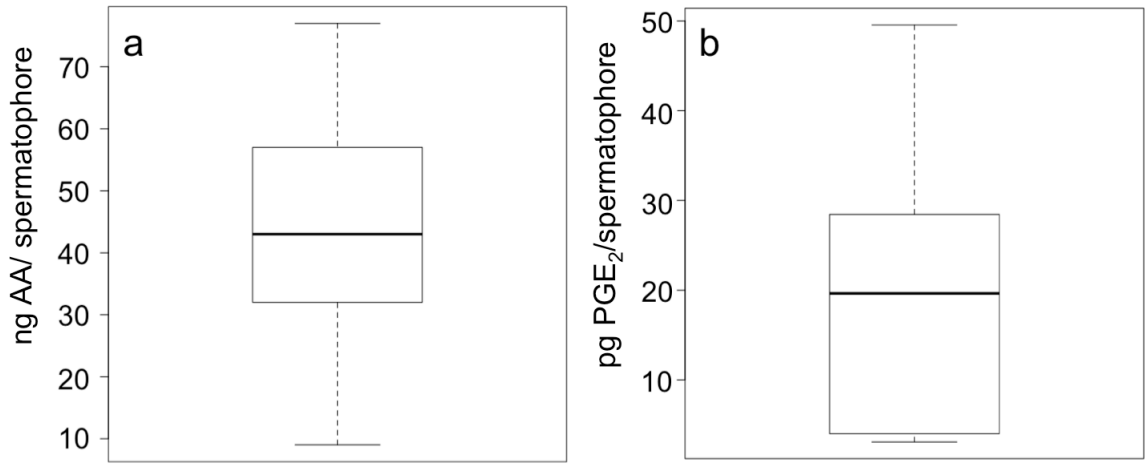

Figure 1. Boxplots showing the average quantity of (a) arachidonic acid (AA) and (b) prostaglandin E_2 (PGE_2) detected in the spermatophores of males. Boxes represents the lower (25%) and upper (75%) quartiles, the solid dark horizontal line is the median, and the whiskers indicate 1.5 times the interquartile range.

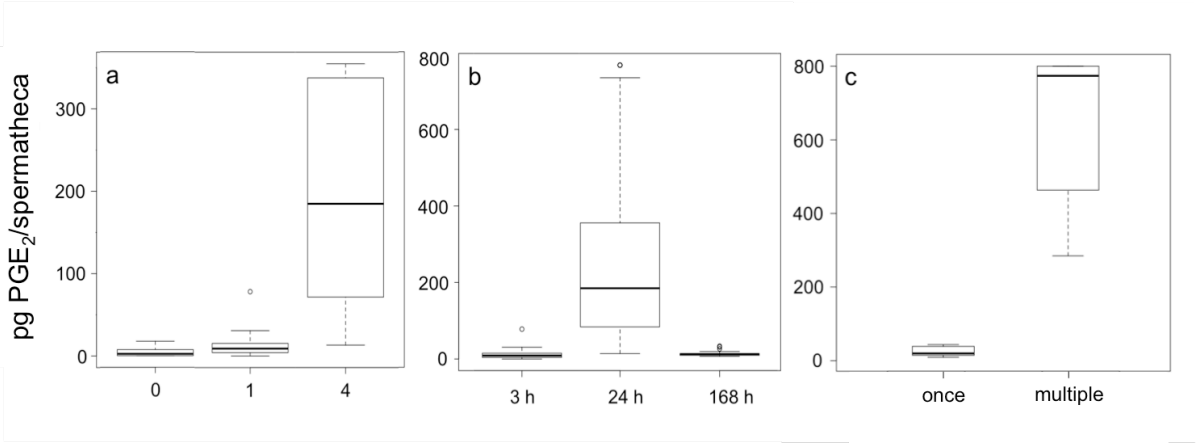

Figure 2. Boxplots showing differences in the quantity of PGE_2 (pg) detected in the spermatheca of females in different experimental mating treatments. Boxplots are shown for spermathecae from females: (a) mated 0, 1 or 4 times then analyzed after 3 h; (b) mated once then analyzed 3, 24, and 168 h post-copula; and (c) mated once at the beginning or multiple times throughout the trial and then analyzed at hour 168 of trial. Boxes represent the lower (25%) and upper (75%) quartiles, the solid dark horizontal line is the median, and the whiskers indicate 1.5 times the interquartile range. Data beyond the end of the whiskers are outliers and plotted as open dots.

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CHAPTER IV

FEMALES GAIN DIRECT BENEFITS FROM IMMUNE-BOOSTING EJACULATES

A paper submitted for publication to a peer-reviewed journal
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Abstract

Females in many animal taxa incur significant costs from mating in the form of injury or infection, which can drastically reduce fecundity and survival. Therefore, immune function during reproduction plays an important role in determining a female's lifetime fitness. Trade-offs between reproduction and immunity have been extensively studied, yet a growing number of studies demonstrate that mated females have stronger immune response than virgins. Here, we use the Texas field cricket, *Gryllus texensis,* to test multiple hypotheses proposed to explain this post-mating increase in immune function. Using host-resistance tests, we found that sexual maturity, copulation, and accessory fluids from the ejaculate do not affect female immunity, but females that acquired ejaculates containing testes-derived components exhibited a significant increase in survival after exposure to a bacterial pathogen. Our data suggest that ejaculatory compounds originating from the testes and transferred to females during sex are responsible for the increased immune function characteristic of mated female crickets.

Introduction

Mating can result in physical injuries and can drastically increase rates of parasitism, predation, and disease (Arnqvist & Nilsson 2000). All of these costs can result in premature death, making the ability of the body to fight off infection or repair wounds an important aspect of reproduction. This is especially true for females, where fitness is often increased by prolonging survival to afford additional time to produce and care for more offspring (Bateman 1948, Trivers 1972). The ties between reproduction and immunity have been extensively studied (Morrow & Innocenti 2011), although most studies focus on how these two life-history traits trade-off with one another, where an increased investment in one results in a decreased investment in the other (Kelly 2011, Bascuñán-García et al. 2010).

Alternatively, there are a growing number of studies indicating that mated females have stronger immune systems than virgin females across a number of taxa (Morrow & Innocenti 2011). For example, field crickets (*Gryllus*) experience increased survival in host resistance tests after mating (Shoemaker et al. 2006) and mating multiple times increases female lifespan (Wagner et al. 2001). Several hypotheses have been proposed to explain this trend of increased immunocompetence after mating, yet remain untested. One possibility is that because mating increases the rates of infection (Knell & Webberly 2004) and physical injury (Crudgington & Siva-Jothy 2000), natural selection has resulted in sexually mature females up-regulating their own immune function prior to mating as a measure to prevent infections that could decrease longevity (Rolff 2002). In contrast, female immunity may increase only after copulation in direct response to an injury or infection obtained during mating (Lawniczak et al. 2007). In such cases, the prolonged physical contact with a male

causes physical damage to be inflicted or pathogens to be transferred to the female, thereby activating the female's immune system.

An alternative explanation could be that elements comprising the ejaculate effect changes in female physiology after mating. In *Gryllus* crickets, accessory gland proteins (*Acps*) have known roles in immune function and defense (Braswell et al. 2006). For example, *Acps* can up-regulate antimicrobial gene expression (McGraw et al. 2004) or have intrinsic antimicrobial activity (Mueller et al. 2007). If responsible, then females that receive *Acps* from an ejaculate will exhibit increased in immune function, even when other ejaculate components (i.e. testes-derived compounds) are absent. Finally, sperm or other components derived from the testes could increase in immune activity. Females may react to sperm as non-self particles, although evidence for this is limited to mammals (Morrow & Innocenti 2011). Alternatively, prostaglandin is a major mediator of the invertebrate immune system (Stanley et al. 2009) and is present in the seminal fluid of crickets (Destephano & Brady 1977). In *Gryllus*, prostaglandin is derived from the testes and is transferred to and accumulated by the female during mating and could therefore increase female immune response after mating.

Here, we empirically test the hypotheses regarding the post-mating boost in female immune response using the Texas field cricket, *G. texensis*. Their courtship is highly ritualized and easily manipulated, allowing for the direct testing of the different proximate mechanisms proposed for the increased immune activity after mating. We experimentally manipulated courtship, copulation, and ejaculate composition to test whether the increased disease resistance exhibited by mated female *G. texensis* is due to a self-regulated increase in immune responsiveness prior to mating, a direct response to infection or trauma sustained

from sexual interactions with males, or the result of ejaculatory compounds derived from the accessory glands or testes.

Methods

Experimental animals

Experimental crickets were lab-reared descendants of mature individuals collected in Austin, TX (USA) in August 2013. Crickets were reared in large communal bins (73 × 41 × 46 cm) until their penultimate instar, after which they were isolated in clear plastic 250 ml containers and checked daily for eclosion. All crickets were housed in an environmentally-controlled room (27 °C; 12:12 h light:dark cycle) and supplied with cotton-plugged water vials and dry cat food (Special Kitty Premium Cat Food) *ad libitum*.

Experimental mating

We randomly assigned adult females (10 days post-eclosion) to one of four treatments where they experienced: 1) courtship, 2) copulation, 3) accessory fluids, or 4) testes-derived components. Pairs of crickets were transferred to a clear plastic mating arena (10 cm diameter × 4.5 cm depth) under a 25-W red light.

To test the hypothesis that the increased immune function is a self-regulated increase, females in the courtship treatment were monitored for sexual behavior to ensure sexual receptivity. When a female mounted her partner, we gently shook the container to deter copulation and immediately removed the male (n = 25). To test whether infection or injury is responsible, females were allowed to copulate, but once a spermatophore was successfully transferred to the female we immediately removed it and separated the pair (n = 18). To test

the effect of accessory gland fluids and *Acps*, we paired females with castrated males. Males were castrated following the procedure detailed in (Larson et al. 2012), which removes testes-derived components (i.e. sperm and prostaglandin) from the ejaculate while leaving accessory fluids intact. Once this accessory-fluid only spermatophore was transferred to the female, we removed the male and allowed the spermatophore to remain attached for 45 min (n = 20). Finally, to test the effect of the testes-derived components of the ejaculate, we paired females with un-manipulated males that could provide an intact ejaculate and allowed them to copulate, after which the male was removed and we allowed the spermatophore to remain attached for 45 min before removal (n = 24). Females were then transferred to individual containers (16.5 × 10.5 × 7 cm) and supplied food and water.

Host resistance

Three days after mating, we subjected females to a host resistance test to determine each female's relative disease resistance, a proxy for overall immune competence (Adamo 2004). Crickets were cold-anesthetized at −20 ºC and pronotum length was measured. We then injected an LD_{50} dose (1.0×10^4 cells/2 µl) of the bacterium, *Serratia marcescens,* into the abdomen using a sterilized glass microcapillary needle. *S. marcescens* is a Gram-negative bacterium lethal to *G. texensis* (Kelly & Tawes 2013) and is commonly found in their natural environment. After injection, we transferred females to clean containers supplied with water and food. Individuals were observed until they fully recovered from the anesthesia, then monitored for mortality every 12 h for the next five days.

Statistics

Statistical analyses were conducted using R (R Development Core Team 2009) with a = 0.05 and visualized using *ggplot2*. We used a Cox regression to test the effect of treatment on survival (Cox 1972, Fox 2002).

Results

We found a significant effect of treatment on survival (Fig. 1), where females that received an ejaculate containing testes-derived components survived significantly longer than females that experienced only courtship (Cox regression: $z = 3.789$; $p = 0.0001$; $n = 50$), copulation with no spermatophore transfer (Cox regression: $z = 4.086$; $p < 0.0001$; $n = 43$), or transfer of a spermatophore containing only accessory fluids (Cox regression: $z = 4.303$; $p < 0.0001$; $n = 45$).

Discussion

We found that ejaculatory compounds derived from the testes are responsible for the increase in host resistance of mated female crickets. These results are consistent with the hypothesis that ejaculates enhance disease resistance in mated females (Domanitskaya et al. 2007). Our results suggest that prostaglandin in the ejaculate, which is well known for its roles in female reproductive investment in crickets (Stanley-Samuelson & Loher 1986) and for its role in modulating the immune response in insects (Stanley et al. 2009) likely alters female immune function after mating.

Exactly how and why this post-mating increase in immunocompetence evolved is unknown. Increased disease resistance after mating may play an important role in countering

the high risk of infection or injuries obtained while mating, thereby allowing females to survive longer and produce more offspring. It is possible then, that increased survival is an ejaculate-derived benefit that offsets the costs of mating. If females indeed obtain fitness benefits, then this could contribute to why female field crickets frequently mate when they receive no other direct benefits from males.

Alternatively, ejaculates can negatively affect female fitness due to sexual conflict. Immune activation is costly and results in tradeoffs with reproduction (Bascuñán-García et al 2010), therefore, up-regulating the immune system in the absence of infection may be detrimental to females. In field crickets, infected females adaptively respond to infection by increasing oviposition rates (Adamo 1999). Immune activation may signal the possibility of a decreased lifespan, resulting in the immediate increase in reproductive effort as a form of terminal investment. This would benefit males as females would begin laying eggs shortly after mating with them, yet could be detrimental to females that overinvest in current reproduction at the expense of future reproduction or survival. However, females selectively invest more in some components of immunocompetence (e.g. phenoloxidase activity) at the onset of sexual maturity (Kelly & Tawes 2013), and are able to maintain high rates of oviposition even after exposure to multiple infections when nutrition is plentiful (Shoemaker & Adamo 2007), suggesting that increased disease resistance may not have the detrimental effects on reproductive investment that life-history theory might predict.

In conclusion, we show that although changes in female physiology after mating are often linked to the action of *Acps*, alternative ejaculate components can be responsible. We demonstrate that prostaglandin, a compound that stimulates egg production and oviposition in crickets, may increase immune activity of mated female organisms. We highlight that

although the physiological effects of testes-derived components on females may have arisen out of sexual conflict, it has resulted in increased female immunocompetence and therefore may help counteract the survival costs that pathogens or injuries inflict on females during the breeding season.

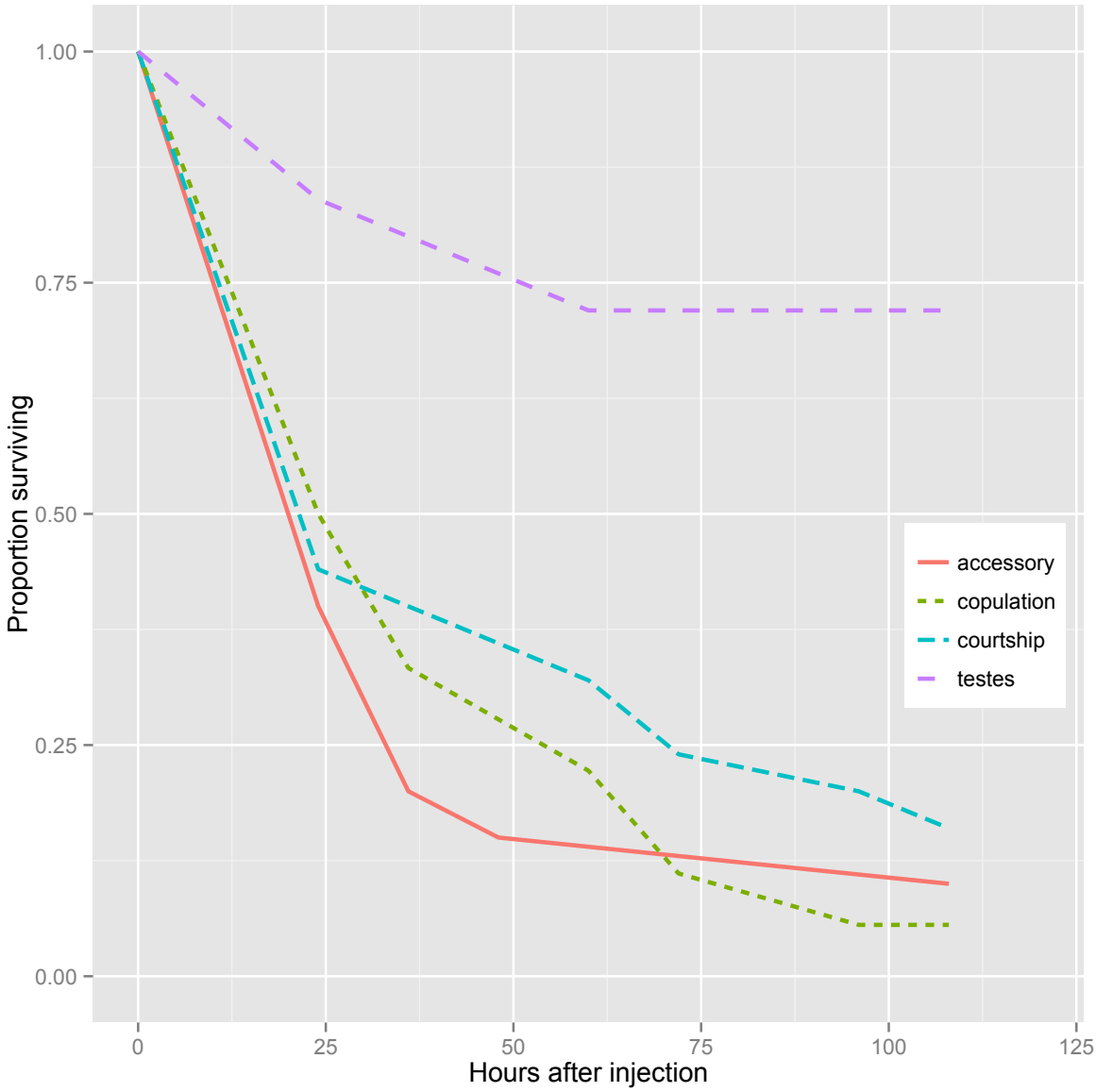

Figure 1. Treatment-specific survival after infection of female crickets experienced courtship (n = 25), copulation (n = 18), accessory fluids (n = 20), or testes-derived components

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CHAPTER V

GENERAL CONCLUSIONS

Sex has significant costs associated with it, yet females of numerous species mate more than is necessary to ensure reproductive success. High female mating rates are even widespread throughout taxa in which males provide no obvious benefits to females upon copulation. Although multiple mating may result in increased fecundity, trade-offs with surviving pathogens or healing injuries incurred during mating are complex. Why females mate at such high rates when the costs to survival are great remains a leading question in behavioral ecology and the e volution of mating systems. In this dissertation, I empirically tested multiple hypotheses about the costs and benefits of female multiple mating to explore the evolution of high mating rates and the consequences they have on female fitness in the Texas field cricket, *Gryllus texensis*.

In chapter 2, I experimentally tested the hypothesis that multiple mating increases female fitness by allowing females to produce more offspring for a longer period of time. I found that higher rates of female mating significantly increased lifetime fecundity independent of whether females mated with one or two males. Further, although interactions with males significantly increased rates of injury or death, females that mated multiple times throughout their reproductive period experienced increased rates and duration of oviposition, demonstrating that the immediate benefits of multiple mating may greatly outweigh the costs that mating poses to female condition and survival. My results suggest that replenishing

ejaculate-derived components is a driving factor of high mating rates in species in which females do not receive external direct benefits from mating.

Because high mating rates and ejaculate replenishment indeed increase female fitness, I predicted that the proximate mechanism responsible is: 1) found in the ejaculate of males, 2) transferred to females during mating, 3) ephemerally available and requires replenishment, and 4) can be accumulated by mating at high rates. Although sperm is an obvious component that might be responsible, there are a number of alternative ejaculate-derived compounds that could mediate post-mating physiology in females. In chapter 3, I focused specifically on prostaglandin E_2 (PGE_2), an oxygenated metabolite of a C20 polyunsaturated fatty acid that is frequently discussed as a potential mechanism for increased female fitness after mating in crickets. To determine whether testes-derived PGE_2 indeed fits the predictions listed above, I directly measured the quantity of PGE_2 in spermatophores and spermathecae of virgin and mated crickets. I found that *G. texensis* males provide both PGE_2 and its precursor arachidonic acid in their ejaculates and that these components are transferred to the female during mating and then stored within the spermathecae. Further, PGE_2 indeed decreases in quantity as time since mating increases, yet female *G. texensis* crickets can maintain continual access to PGE_2 by frequently mating. I hypothesize that the continual need for PGE_2 in part explains the high mating rates observed in female crickets and could proximately explain the positive relationships between fitness and mating rate.

Although females gain significant fecundity benefits from mating at high rates, my results from chapter 2 show that they also incur significant costs from mating in the form of injury or infection. Therefore, strong immune function likely plays a major role in extending female survival during the breeding season. A growing number of studies demonstrate that

mated females have stronger immune response than virgins, and in chapter 4, I tested multiple hypotheses proposed to explain the post-mating increase in immune function. Using host-resistance tests, I found that onset of sexual maturity, the act of copulation, and components within the accessory fluid of the ejaculate do not alter female *G. texensis* immunity. Alternatively, intact ejaculates significantly increased a female's ability to survive a pathogenic bacterial infection. My results show that ejaculatory compounds originating from the testes and transferred to females during copulation are responsible for the increased immune function characteristic of mated female crickets. These data support the hypothesis that mating-derived PGE_2, which originates from the testes and is absent from the ejaculates of castrated males, is likely responsible.

In summary, my research demonstrates that although mating has survival costs associated with it, female field crickets derive significant fitness benefits from mating at high rates. Mating not only increased lifetime fecundity, but also increased disease resistance, a direct benefit obtained from testes-derived components within the ejaculate. Although I found support that PGE_2 is responsible for these post-mating changes in female physiology, additional experiments directly separating the effects of sperm and PGE_2 are needed in order to confirm this proximate mechanism. In light of these findings, I suggest that the continual need for PGE_2 is in some respect responsible for the high mating rates observed in female crickets and could proximately explain the positive relationship between fitness and mating rate. Because male investment in ejaculate size and composition (e.g. relative amount of sperm, accessory fluids, seminal fluid proteins, etc.) varies significantly across orthopteran species, a comparative examination of female mating rates and ejaculate composition may provide additional insight into what role PGE_2 plays in the evolution of female mating rates.

If the quantities of PGE_2 that females obtain from ejaculates indeed correlate with species-specific mating rates, it could explain why females from species that do not obtain obvious direct benefits from mating (e.g. nutritional gifts or parental care) still experience increased fitness despite the costs of sex. Further, because PGE_2 is a major mediator of immunity and is commonly found in ejaculates of many animal taxa, it could explain the wide-ranging pattern of increased immune function that females exhibit after mating.

APPENDIX A

EFFECT OF PGE_2 AUGMENTATION AND INHIBITION ON OVIPOSITION

Objective

Prostaglandin is a known mediator of the insect reproductive system (Stanley 2006) and a common practice in examining its role in female fecundity and oviposition is to manipulate the levels of prostaglandin in the body by directly injecting pharmacological doses of PGE_2 or cyclooxygenase pathway inhibitors (e.g. dexamethasone) into the organism. Several past studies have used this technique to understand the effect that PGE_2 has on female fecundity in *Teleogryllus oceanicus* (Loher 1979, Loher et al. 1981, Stanley-Samuelson, 1986) and *Acheta domesticus* (Destephano & Brady 1977, Murtaugh & Denlinger 1987, Tanaka 2014), with results demonstrating that administering larger quantities of PGE_2 results in a dose-dependent increase in oviposition rate. If prostaglandin indeed mediates female reproductive investment after mating in female *Gryllus texensis*, then virgin females injected with PGE_2 should exhibit oviposition rates equal to that of mated females. Alternatively, mated females injected with dexamethasone should have oviposition rates equal to that of virgin females due to the inhibition of post-mating synthesis of PGE_2. Here, we examine the effects that direct augmentation or inhibition of PGE_2 has on oviposition rates in the field cricket, *Gryllus texensis*.

Methods

Experimental crickets were lab-reared descendants of mature individuals collected in Austin, TX (USA) in October 2010. Crickets were reared in large communal bins (73 × 41 ×

46 cm) until their penultimate instar, after which they were isolated in clear plastic 250 ml containers and checked daily for eclosion. All crickets were housed in an environmentally-controlled room (27 °C; 12:12 h light:dark cycle) and supplied with cotton-plugged water vials and dry cat food (Special Kitty Premium Cat Food) *ad libitum*.

We randomly assigned adult females (8 days post-eclosion) to one of two mating treatments: virgin or mated. All females were placed in a clean deli cup and handled equally. Females in the mated treatment were paired with a virgin male and allowed 60 minutes to mate. If the pair did not mate after 60 minutes, an alternative male was provided. If a female did not mate one of three consecutive males, the trial was cancelled and the female was removed from the experiment. Alternatively, after a successful mating, males were immediately removed to prevent additional copulation. After the mating trials, mated and virgin females were assigned to one of three treatments where they were injected with: 1 µl of phosphate buffered saline (PBS) solution, 10 µg/1 µl of prostaglandin E_2 (PGE_2) dissolved in a 1:5 ethanol:PBS solution, or 20 µg/1 µl dexamethasone (cyclooxygenase inhibitor) dissolved in a 1:5 ethanol:PBS solution. Crickets were cold-anesthetized at −20 ºC and injections of the assigned solution (PBS, PGE_2, or dexamethasone) were delivered near the common oviduct region in the membrane between the genital plate and last abdominal segment using a sterilized glass microcapillary needle. After injection, females were transferred to individual containers and supplied with water and food *ad libitum* and a small petri dish of moist sand. Females were observed until they fully recovered from the anesthesia. After 24 h, the number of eggs laid in the sand were quantified and the female was provided with another container of moist sand. For 9 days, females were allowed to lay eggs in this sand, after which fresh sand was provided and the eggs laid within the next 24 h

were again quantified to determine whether the mating or injection treatment significantly affected the duration of oviposition exhibited by females.

Results

The results from our experiment are in the table below. We did not conduct formal statistical analyses on these data due to small sample sizes, an excess of zeroes, and overdispersion; however, several trends can be observed. First, mated females laid more eggs than virgin females irrespective of injection treatment. Second, administering PGE_2 to virgin females did not initiate oviposition, and although mated females that received a dose of PGE_2 did lay a larger number of eggs on average than mated females that received the control injection, the variation around the means was too large to see a clear effect. Finally, dexamethasone did not inhibit oviposition in mated females, as demonstrated by these females laying equal numbers of eggs as mated females in the injected with the control solution of PBS.

Table 1. Effect of mating and injected prostaglandin on oviposition rates

Mating treatment	Injection treatment	N	Pronotum width (± sd)	Eggs laid 1 day after mating (± sd)	Eggs laid 9 days after mating (± sd)
Virgin	PBS	17	3.28 ± 0.29	0.1 ± 0.2	2.0 ± 3.6
Virgin	Dexamethasone	9	3.39 ± 0.34	0.2 ± 0.4	9.0 ± 22.0
Virgin	PGE_2	13	3.35 ± 0.31	0.2 ± 0.6	0.5 ± 1.0
Mated	PBS	11	3.21 ± 0.40	7.7 ± 19.0	27.9 ± 38.1
Mated	Dexamethasone	19	3.39 ± 0.33	6.9 ± 17.1	32.5 ± 40.2
Mated	PGE_2	12	2.93 ± 0.92	18.5 ± 16.6	40.7 ± 45.2

Discussion

Our results suggest that PGE_2 alone does not mediate oviposition in field crickets. Contrary to prediction, injection of PGE_2 did not stimulate virgin females to oviposit eggs at a rate higher than the control virgin females. Mated females treated with PGE_2 did exhibit a slight increase in oviposition rate, however, so prostaglandin in addition to other mating-derived stimuli may help to mediate female fecundity. We saw no effect treating either virgin or mated females with the cyclooxygenase inhibitor, dexamethasone. Several past studies have found significant effects prostaglandin and prostaglandin synthesis inhibitors on female fecundity in crickets, so below we discuss several factors that may have contributed to the difficulty in reproducing these effects to help other scientists avoid the same pitfalls that we encountered.

First, although we observed the male and female pairs to ensure that a spermatophore was transferred, we did not check the spermathecae of females after the trials were over to ensure that an ejaculate had indeed been acquired. There is a surprisingly high rate of mating failure in crickets and this may have contributed to the large variation in number of eggs laid within mated females. Additionally, females exhibit cryptic female choice as to how long they allow a spermatophore to remain attached. As attachment duration increases, so too does the quantity of ejaculate transferred to the female. Because we did not control for attachment duration, the unstandardized quantities of ejaculates acquired by females could have contributed to the variation in oviposition rates.

Second, we tried to minimize the toxicity of the solutions we injected by dissolving the PGE_2 or dexamethasone in a very small amount of ethanol, then diluting in PBS. We believe that this method did not dissolve the powders fully into solution, which may have

affected both the dose that each cricket received and whether the solution was actually incorporated into the body. If we were to repeat this experiment, we would dissolve the substances in a larger quantity of ethanol and then slowly reduce its volume to keep it in solution, or we would use a stronger solvent such as DMSO.

Finally, previous research by Loher (1981) demonstrated that pharmacological doses of PGE_2 or dexamethasone need to be administered in order to see an effect on oviposition due to how quickly the compounds are metabolized by the body; however, direct application to the genital chamber drastically reduces the quantity required to stimulate egg laying. Therefore, we chose to inject a smaller quantity to the genital chamber as opposed to injection into the main hemocoel of the cricket. Administering treatments this way occasionally resulted in some of the solution being excreted from the genital opening, which may have played a role in the large variation in the number of eggs that females in each treatment laid. Injection directly into the abdomen may have had a shorter-lasting effect on female physiology, but would have more accurately standardized the dose within each treatment.

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APPENDIX B

EFFECT OF PROSTAGLANDIN E_2 AND $F_{2\alpha}$ ON HOST RESISTENCE

Objective

Many past studies have manipulated the levels of prostaglandin within the insect body to examine its effects on components of immunity (e.g. Mandato et al. 1997, Jurenka et al. 1999, Carton et al. 2002, Shrestha & Kim 2009). Prostaglandin is a known mediator of the insect immune system (Stanley et al. 2009), such that administering larger quantities of prostaglandin results in a dose-dependent increase in nearly all arms of the immune response. Here, we inject physiological doses of two forms of prostaglandin, E_2 and $F_{2\alpha}$, to examine the effect that they have on the survival of the field cricket, *Gryllus texensis*, to an infection by a bacterial pathogen.

Methods

Experimental crickets were lab-reared descendants of mature individuals collected in Austin, TX (USA) in October 2010. Crickets were reared in large communal bins (73 × 41 × 46 cm) until their penultimate instar, after which they were isolated in clear plastic 250 ml containers and checked daily for eclosion. All crickets were housed in an environmentally-controlled room (27 °C; 12:12 h light:dark cycle) and supplied with cotton-plugged water vials and dry cat food (Special Kitty Premium Cat Food) *ad libitum*.

We randomly assigned adult females (7-9 days post-eclosion) to one of three treatments where they were injected with 4 µl of: 1) 1:3 ethanol:phosphate buffered saline (PBS) solution, 2) 100 µg prostaglandin E_2 (PGE_2) dissolved in a 1:3 ethanol:PBS solution,

or 3) 100 µg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) dissolved in a 1:3 ethanol:PBS solution. Each female was subjected to a host resistance test to determine each their relative disease resistance, a proxy for overall immune competence. Crickets were cold-anesthetized at −20 ºC and pronotum length was measured. We injected the assigned solution (PBS, PGE_2, or $PGF_{2\alpha}$) into the right side of the abdomen and immediately injected an LD_{50} dose (1.0×10^4 cells/2 µl) of the bacterium, *Serratia marcescens,* into the left side of the abdomen using a sterilized glass microcapillary needle. After injection, we transferred females to clean containers supplied with water and food. Individuals were observed until they fully recovered from the anesthesia, then monitored for mortality every 24 h for the next five days. Statistical analyses were conducted using R (R Development Core Team 2009) with a = 0.05 and visualized using *ggplot2*. We used a Cox regression to test the effect of treatment on survival.

Results

We did not find a significant effect of treatment on survival. Females injected with PGE_2 (Cox regression: z = 1.455; p = 0.146; n = 38) or $PGF_{2\alpha}$ (Cox regression: z = 0.032; p = 0.974; n = 38) did not survive significantly longer than females that were injected with the control solution.

Table 1. Effect of PGE_2 and $PGF_{2\alpha}$ on LD_{50} host resistance tests

Treatment	Sample size	Pronotum ± se	% Survival	Average Day of Death
PBS	17	3.33 ± 0.06	35.29 %	1.27 ± 0.11
PGE_2	13	3.19 ± 0.07	7.69 %	1.23 ± 0.14
$PGF_{2\alpha}$	8	3.37 ± 0.07	37.50 %	1.20 ± 0.16

Discussion

Our results suggest that injection of prostaglandin does not significantly increase the ability to survive infection of a bacterial pathogen in field crickets. Contrary to prediction, injection of $PGF_{2\alpha}$ had no effect relative to the control group, and injection of PGE_2 actually had a slight, although non-significant effect of reducing survival. Other studies have found significant effects prostaglandin on the cricket immune system, where is increases both the nodulation of bacterial pathogens (Miller et al. 1999) and the melanization of a nylon filament (Tanaka 2014).

There are several confounding factors that may have contributed to these results, which is why they were not included within the main chapters of this dissertation. We include discussion of some of these issues here to assist future research in this area and help other scientists avoid the same pitfalls that we encountered. First, crickets were randomly assigned to one of the three treatments, and by chance, the average size of the cricket within the PGE_2 treatment was smaller than those assigned to the other two treatment groups. This size discrepancy could have contributed to the trend of crickets injected with PGE_2 exhibiting reduced survival because they received a larger dose of bacteria for their body size.

One other confounding factor was the skill with which the doses of PBS or prostaglandin were administered. The vast majority of crickets died within 24 hours of injection of a bacterial pathogen and many did not recover from the injection itself (and hence were not included in the analysis). Later attempts to inject crickets with bacteria (see chapter 4) did not result in the immediate death of any individuals, suggesting that when administered properly, the effect of the injection itself should be minimal. Further, we only observed the crickets once daily for mortality. This time frame is not fine-scale enough to

provide the detail required to be able to distinguish differential death rates between the treatments. We suggest checking mortality a minimum of every 12 hours to obtain an appropriate survival curve of the subjects involved.

Finally, we tried to minimize the toxicity of the solutions we injected by dissolving the prostaglandin in a very small amount of ethanol, then diluting in PBS. We believe that this method did not dissolve the powdered prostaglandin fully into solution, which may have affected both the dose that each cricket received and how the prostaglandin was actually incorporated into the body. If we were to repeat this experiment, we would dissolve the prostaglandin in a larger quantity of ethanol and then slowly reduce its volume to keep it in solution, or we would use a stronger solvent such as DMSO.

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